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SIRI TÄHTINEN

Combining Oncolytic Immunotherapies to Break Tumor Resistance

CANCER GENE THERAPY GROUP
DEPARTMENT OF PATHOLOGY
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COMBINING ONCOLYTIC IMMUNOTHERAPIES TO BREAK TUMOR RESISTANCE

Siri Tähtinen

Cancer Gene Therapy Group

Department of Pathology

Doctoral Programme in Biomedicine (DPBM)

Faculty of Medicine

University of Helsinki

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Supervised by

Akseli Hemminki, MD, PhD
Professor of Oncology
Cancer Gene Therapy Group,
Department of Pathology,
Faculty of Medicine,
University of Helsinki, Finland

Markus Vähä-Koskela, PhD, Docent
Institute for Molecular Medicine Finland,
University of Helsinki, Finland

Reviewed by

Hanna Jarva, MD, PhD, Docent
Department of Bacteriology and Immunology,
University of Helsinki, Finland

Kari Airenne, PhD, Adjunct Professor
Head of Scientific Projects
FinVector Vision Therapies Oy, Finland

Official opponent

Manuel J.T. Carrondo, PhD
Professor of Chemical and Biochemical Engineering
Departamento de Química, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Portugal
Instituto de Biologia Experimental e Tecnológica (IBET), Portugal

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“In the fields of observation, chance favors only the prepared mind”

– Louis Pasteur

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PART A

LIST OF ORIGINAL PUBLICATIONS

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II. **TÄHTINEN S**, Grönberg-Vähä-Koskela S, Lumen D, Merisalo-Soikkeli M, Siurala M, Airaksinen AJ, Vähä-Koskela M, Hemminki A. (2015). Adenovirus Improves the Efficacy of Adoptive T-cell Therapy by Recruiting Immune Cells to and Promoting Their Activity at the Tumor. Cancer Immunol Res. (8):915-25.

III. **TÄHTINEN S**, Kaikkonen S, Merisalo-Soikkeli M, Grönberg-Vähä-Koskela S, Kanerva A, Parviainen S, Vähä-Koskela M, Hemminki A. (2015). Favorable Alteration of Tumor Microenvironment by Immunomodulatory Cytokines for Efficient T-Cell Therapy in Solid Tumors. PLoS One. 10(6):e0131242.

IV. Vähä-Koskela M, **TÄHTINEN S**, Saha D, Liikanen I, Hemminki O, Taipale K, Grönberg-Vähä-Koskela S, Merisalo-Soikkeli M, Diallo J-S, Kanerva K, Cerullo V, Hemminki A. Blocking innate defense signaling improves oncolytic adenovirus efficacy in virus-resistant ovarian carcinoma. Manuscript.

ABBREVIATIONS

aAPC	artificial antigen-presenting cell
ACT	adoptive (T-) cell therapy
Ad	adenovirus
ADCC	antibody-depended cell-mediated cytotoxicity
AIDS	Acquired Immune Deficiency Syndrome
ALL	acute lymphoblastic leukemia
APC	antigen-presenting cell
ATAP	Advanced Therapy Access Program
BiTE	bi-specific T-cell engager
BSA	bovine serum albumin
BSL-2	biosafety level 2
BTLA	B- and T-lymphocyte attenuator
CAR	chimeric antigen receptor
CAR-T	chimeric antigen receptor T-cell
CD40L	CD40 ligand
CDC	complement-depended cytotoxicity
CEA	carcinoembryonic antigen
CLL	chronic lymphocytic leukemia
CMV	cytomegalovirus
CR	complete response
CT	computed tomography
CTL	cytotoxic T-lymphocyte
CTLA-4	CTL-associated protein 4
CRS	cytokine release syndrome
DAMP	damage-associated molecular pattern
DC	dendritic cell
DMEM	Dulbecco's Modified Eagle's medium
DSG-2	desmoglein 2
EEV	extracellular enveloped virus
ELISPOT	Enzyme-Linked ImmunoSpot
EM	electron microscope
EMA	European Medicines Agency
EpCam	epithelial cell adhesion molecule
FCS	fetal calf serum

FDA	Food and Drug Administration
FRC	fibroblastic reticular cell
GFP	green fluorescent protein
GM-CSF	granulocyte macrophage -colony stimulating factor
gp100	glycoprotein 100
hCAR	human coxackie and adenovirus receptor
HER-2	human epidermal growth factor receptor 2
hGM-CSF	human GM-CSF
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
IDO	indoleamine 2,3-dioxygenase
IFN	interferon
IL	interleukin
IMV	intracellular mature virus
IVIS	*in vivo* imaging system
JAK	Janus kinase
LAG-3	lymphocyte-activation gene 3
mAb	monoclonal antibody
MART-1	melanoma antigen recognized by T cells 1
MAGE-A3	melanoma-associated antigen A3
MDSC	myeloid-derived suppressor cell
MHC I	major histocompatibility complex class I
NAb	neutralizing antibody
NK	natural killer
NY-ESO1	New York esophageal squamous cell carcinoma 1 (antigen)
OVA	ovalbumin
PAMP	pathogen-associated molecular pattern
PDAc	pancreatic ductal adenocarcinoma
PD-1	programmed (cell) death 1
PD-L1	programmed (cell) death ligand 1
PFU	plaque forming unit
PR	partial response
qPCR	quantitative (real-time) polymerase chain reaction
Rb	retinoblastoma
REP	rapid expansion protocol
RPMI	Roswell Park Memorial Institute medium

SAE	severe adverse event
scFv	single-chain variable fragment
SCID	severe combined immunodeficiency
SD	stable disease
SFV	Semliki Forest virus
SPECT	single-photon emission computed tomography
TAA	tumor-associated antigen
TAM	tumor-associated macrophage
TAN	tumor-associated neutrophils
TBI	total body irradiation
T_{CM}	central memory T-cell
TCR	T-cell receptor
tdLN	tumor-draining lymph node
T_{EM}	effector memory T-cell
TGF	transforming growth factor
Th	T helper (cell)
TIL	tumor-infiltrating lymphocyte
TIM-3	T-cell immunoglobulin and mucin-domain containing 3
TK	thymidine kinase
TLR	Toll-like receptor
TME	tumor microenvironment
TNF	tumor necrosis factor
Treg	T regulatory cell
TRP-2	tyrosinase related protein 2
TRUCK	T-cells re-directed for universal cytokine-mediated killing
T-VEC	Talimogene laherparevec (hGM-CSF armed Herpes simplex virus)
VCP	virus complement protein
VGF	vaccinia growth factor
VP	viral particle
VSV	vesicular stomatitis virus
VV	vaccinia virus
WR	Western Reserve (strain of vaccinia virus)

ABSTRACT

According to latest estimates, cancer is becoming an increasing health risk on a global scale. Consequently, novel cancer treatment modalities are urgently needed, especially for the treatment of metastatic solid tumors that are refractory to standard therapies. One promising approach in the treatment of such advanced cancers is immunotherapy which aims to elicit *de novo* immune responses and/or to boost pre-existing anti-tumor immunity. Different forms of cancer immunotherapy include oncolytic viruses, which selectively replicate in and destroy cancer cells, and adoptive T-cell therapy, in which the patient is given vastly amplified numbers of tumor-targeting T-cells. Both of these have shown capacity to elicit anti-tumor immunity but efficacy in clinical settings has been suboptimal due to different resistance mechanisms employed by solid tumors.

Anti-viral resistance represents a major hurdle in oncolytic virotherapy, as repeated administration of the same virus can lead to induction anti-viral rather than anti-tumor immunity. Moreover, cancer cells in some tumors may intrinsically be resistant to virus infection. In study I, we examined whether this could be circumvented by heterologous prime-boost setting, i.e. by switching between oncolytic adenovirus (Ad) and vaccinia virus (VV) during therapy. The results showed that presence of one virus does not preclude the infection of another and treatment with heterologous Ad-VV therapy can delay the onset of anti-viral resistance. Moreover, we found that restricted replication of the priming (adeno)virus can affect the efficacy of heterologous virotherapy. In study IV, we studied the role of anti-viral signaling in adenovirus replication in cancer cells and whether this could be augmented with Janus Kinase 1/2 inhibitor Ruxolitinib. Interestingly, we found that although exposure to type I interferon does not inhibit progressive Ad replication *in vitro*, significant improvement in anti-tumor efficacy of the virus was observed *in vivo* when combined to concomitant Ruxolitinib treatment. These results underline the possible approaches that could be taken to reduce naturally acquired or therapy-induced resistance, which interferes with viral spread and may hinder the therapeutic efficacy.

Adoptive T-cell therapy (ACT) can be a potent form of immunotherapy. Despite the large number of anti-tumor T-cells infused during ACT, immunosuppression and immune evasion of advanced tumors can render tumor-infiltrating lymphocytes (TILs) inactive. In study II, we examined whether oncolytic adenovirus could increase anti-tumor efficacy of adoptively transferred T-cell receptor (TCR) transgenic T-cells. Indeed, intratumoral injections of adenovirus were able to counteract immunosuppression by activating antigen-presenting cells (APCs) and anti-tumor T-cells. Moreover, an endogenous T-cell response against other, non-related tumor antigens was detected and this polyclonal response contributed to systemic anti-tumor immunity. In study III, we analyzed whether cellular composition of tumor microenvironment could be modified by local administration of immunostimulatory recombinant cytokines. When combined to adoptive T-cell transfer, intratumoral injections of interleukin 2 (IL-2), interferon α (IFN-α) and interferon γ (IFN-γ) resulted in significant anti-tumor efficacy, increased tumor-levels of stimulatory immune cells and reduced exhaustion of CD8+ TILs. In contrast, administration of granulocyte-macrophage colony-stimulating factor (GM-CSF) enhanced tumor growth and recruited immunosuppressive cell types such as monocytic myeloid-derived suppressor cells (MDSCs) and M2 macrophages to the tumor bed. These results indicate that immunomodulation by carefully selected cytokines and/or oncolytic adenovirus can sensitize the tumor in favor of adoptively transferred anti-tumor T-cells.

In conclusion, different combinatorial approaches can be employed to overcome intrinsic, naturally acquired or therapy-induced resistance to anti-tumor T-cells or oncolytic adenovirus. These advances enable significant improvement in the treatment of solid cancers and can potentially lead to development of curative cancer immunotherapies.

PART B

1 REVIEW OF THE LITERATURE

1.1 Introduction

Cancer is a common name for a wide range of malignant diseases that arise from a multistep process involving several genetic and epigenetic alterations, causing the transformed cells to grow uncontrollably. Hallmarks of cancer include self-sufficiency in growth signals, insensitivity to growth inhibitors, limitless proliferative potential, sustained angiogenesis, evasion of apoptosis, formation of tumor-promoting inflammation and the capability to invade tissues to form metastases (Hanahan and Weinberg 2000, Mantovani 2009). The combination of these properties coupled with the complex tumor microenvironment (TME) makes tumors relatively difficult to eliminate, especially in metastasized settings.

According to the recently released Cancer Progress Report 2015 by the American Association for Cancer Research, development of cancer into metastatic disease is the cause of 90 % of cancer-related deaths (cancerprogressreport.org/2015/ on January 15, 2016). Despite the tremendous improvements in cancer prevention, diagnostic techniques and screening procedures, cancer treatment modalities have stayed somewhat the same for decades. With a few exceptions, treatment options for established solid cancers include surgery, radiotherapy and chemotherapy, of which the latter two come at the cost of significant treatment-related side effects.

As the world's population is getting older and risk factors such as obesity, smoking and physical inactivity are getting increasingly common, cancer is becoming an even bigger global challenge. In 2035, an estimated 24 million new cases of cancer will be diagnosed globally, with 14.6 million people predicted to die from the disease

(cancerprogressreport.org/2015/ on January 15, 2016). Consequently, novel cancer therapies are urgently needed, especially for the treatment of metastatic and/or treatment-refractory cancers which respond poorly to conventional treatments.

1.2 Cancer Immunology and Immunotherapy

Cancer immunology, also called onco-immunology, is a branch of immunology which focuses on the spatiotemporal interactions between the immune system and cancer cells. According to the original concept of cancer immunosurveillance introduced in 1957, the immune system has an important role in inhibiting carcinogenesis and maintaining cellular homeostasis (Burnet 1957). This original theory was later expanded into the concept of immunoediting, which also included the paradoxical tumor-sculpting actions of the immune system on developing tumors (Dunn et al. 2002).

Immunoediting contains three distinct phases (elimination, equilibrium and escape), designated the "three E's". In the elimination phase, developing malignant cells are successfully eradicated by the immune system working in concert with intrinsic tumor suppressor mechanisms. The process of elimination includes both innate (such as natural killer (NK) cells and macrophages) and adaptive (T-cell) immune responses against transformed cells, leading to initial repression of nascent tumor. In the equilibrium phase, the host immune system and cancer cell variants surviving from the elimination process enter into a dynamic equilibrium, where anti-tumor T-cells restrain but can't fully eradicate the developing tumor. The equilibrium phase can last several years, during which new immune-resistant tumor cell clones arise due to the strong selection pressure. Finally, in the escape phase, these surviving tumor variants begin to expand in an uncontrolled manner and form a clinically observable malignant tumor that can be fatal to the host if left untreated (Dunn et al. 2002, Kim et al. 2007).

Simultaneously with the growing increase in understanding of tumor immunology, several approaches to harness the immune system to fight cancer have been developed.

The ultimate goal of these therapies, known as cancer immunotherapies, is to generate complete, long lasting remissions and cancer cures by inducing and enhancing patient's own anti-tumor immune responses (Schuster et al. 2006). Moreover, providing that sufficient target specificity is achieved, significant reduction in treatment-related side effects could be envisioned compared to standard treatments. Cancer immunotherapies can roughly be divided into two categories: 1) active immunotherapy (such as oncolytic virotherapy), focuses on generating an immune stimulus within the host and overcoming the reluctance of the immune system to attack the tumor, and 2) passive immunotherapy (such as adoptive T-cell therapy) is based on infusion of tumor-specific antibodies or white blood cells produced and/or modified outside the body (Schuster et al. 2006).

1.3 Oncolytic Viruses

Two types of oncolytic viruses exist: either virus has a natural selectivity towards cancer cells (such as reovirus) or the virus has been genetically engineered to selectively replicate in cancer cells (such as adenovirus or vaccinia virus). In the latter case, modified oncolytic viruses can enter both tumor and normal cells but virus replication and subsequent lysis is restricted to malignant cells which (over)express factors essential for virus replication. Oncolytic viruses constitute a self-amplifying platform for prolonged, local expression of immunostimulatory molecules and sustained presence of virus-mediated danger signals in tumor that can lead to induction of both anti-viral and antitumor immune responses in immunocompetent hosts (Kaufman et al. 2015).

1.3.1 Adenovirus

Adenoviruses (Ad) are one of the most commonly used and studied gene therapy vectors to date. The family of adenoviruses, Adenoviridae, can be divided in 5 genera and 7 species (A-G) containing all together 59 identified serotypes (Liu et al. 2012). Out of these, serotype 5 adenovirus (Ad5) is classified to species C and serotype 3 (Ad3) to

species B based on their ability to agglutinate erythrocytes (Rosen 1960). Species C and B adenoviruses are optimal candidates for oncolytic immunotherapy vectors as they are capable of infecting both dividing and non-dividing cells, followed by efficient replication and lysis of the host cell. Both serotypes are also amenable for genetic engineering of adenoviral capsid and the genome, allowing enhanced infectivity and cell-specific transgene expression. Recombinant replication-competent adenoviruses can accommodate large inserts, for example several immunostimulatory cytokine genes in a single vector (Choi et al. 2012).

1.3.1.1 Structure and Life Cycle

Adenoviruses are non-enveloped, double-stranded DNA viruses covered by icosahedral protein capsid consisting of hexon and penton proteins, latter of which are central for virus internalization (Stewart et al. 1991). Interaction of Ad with target receptor is mediated by fiber protein containing a knob that extends from each vertex of the capsid. Inside the capsid is the linear double-stranded DNA genome and associated core proteins that provide structure and help packaging new virions (Reddy and Nemerow 2014).

The life cycle of adenovirus is divided in early and late phases, the first of which is induced once adenovirus enters the target cell by binding to a high-affinity cell surface receptor such as human coxsackie and adenovirus receptor (hCAR) (Bergelson et al. 1997, Roelvink et al. 1998). Following binding to the primary receptor, interaction between Arg-Gly-Asp (RGD) motif of penton protein and αβ integrins on target cell trigger endocytosis of the virus (Mathias et al. 1994, Roelvink et al. 1999). In the early phase, uncoated virions are transported into the nucleus and gene expression of early transcription cassettes E1A, E1B, E2, E3 and E4 is initiated. The rapid expression of viral E1A protein following virus entry modulates cellular metabolism in favor of virus replication and ensures that the host cell enters the S phase of the cell cycle by binding retinoblastoma protein (pRb) (Whyte et al. 1988a). In subsequent steps, E2 and E3

proteins are involved in viral DNA replication and inhibition of host anti-viral immune responses (Tollefson et al. 1996, Russell 2000), whereas alternatively spliced E4 products promote expression of late viral genes and facilitate viral mRNA metabolism (Halbert et al. 1985, Weigel and Dobbelstein 2000). In the late phase, structural proteins are expressed from transcription cassettes L1-L5 and new virions are assembled. Finally, adenovirus life-cycle culminates in the lysis of host cell, releasing new infectious virions into the extracellular space. This entire cycle is usually completed within 36 hours (Russell 2000).

1.3.1.2 Modifications

Several approaches have been taken to increase the safety and efficacy of adenovirus in oncolytic settings, including both transcriptional and transductional targeting. One of the most important modifications of wild-type Ad has been the 24-base pair deletion (D24) in pRb binding site of the E1A region, which attenuates virus replication in normal cells with wild-type pRb (Fueyo et al. 2000) (**Figure 1**). The inability of D24-modified E1A to bind pRb prevents the release of E2F from pre-existing cellular E2F-pRb complexes and subsequently results in inhibition of E2F-mediated activation of genes associated with both adenoviral E2 promoter and cell cycle regulation in normal cells. In contrast, most cancers presumably have defective pRb/p16 pathway (Whyte et al. 1988b, Sherr 1996, Sherr and McCormick 2002) and the constantly available E2F renders E1A dispensable, thus enabling virus replication in malignant cells (Heise et al. 2000). In addition to genetic deletions, several tumor-specific promoters (such as E2F, Cox-2, VEGF and hTERT) have been designed to exploit the ubiquitous expression of these factors in various cancer types and thus to improve the specificity of oncolytic adenoviruses (Ito et al. 2006, Kanerva et al. 2008, Rojas et al. 2009).

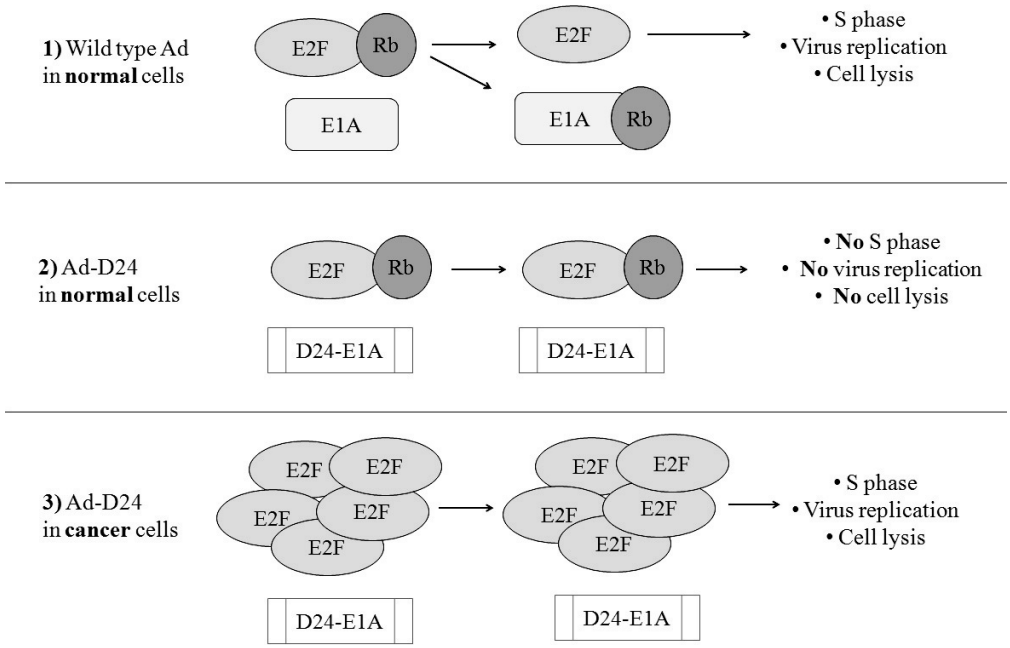

Figure 1. During the replication of wild type adenovirus, E1A binds Rb which is no longer able to repress transcription factor E2F (1). The release of E2F is required for activating genes that promote cell cycle and adenovirus replication. In contrast, adenovirus featuring a D24 deletion (Ad-D24) is unable to bind Rb, subsequently attenuating virus replication in normal cells (2). In cancer cells, D24 is complemented by inactivation of Rb by p16/Rb pathway defects, enabling virus replication and oncolysis (3).

To enhance Ad targeting to cancer cells, adenovirus serotype 5 knob can be replaced with a serotype 3 knob (Krasnykh et al. 1996). These 5/3 chimeric adenoviruses have shown increased gene transfer efficacy in many tumor types (Kanerva et al. 2002, Kangasniemi et al. 2006a, Guse et al. 2007, Bramante et al. 2014), possibly due to high expression of Ad3 receptor desmoglein-2 (DSG-2) on tumor cells (Wang et al. 2011). By contrast, the Ad5 receptor hCAR has been reported to be downregulated in many types of cancer due to Raf-MAPK pathway activation (Anders et al. 2003), underlining the attractiveness of the 5/3 chimeric approach. Other modifications of adenovirus

capsid include incorporation of ligands and motifs (such as RGD and pk7) that bind adhesion molecules on cancer cell surface (Dmitriev et al. 1998, Wu et al. 2002, Kangasniemi et al. 2006b).

1.3.1.3 Immune Responses

1.3.1.3.1 Anti-Viral Responses

Following adenovirus infection, the host immediately recognizes Ad as a "non-self" invading pathogen. This recognition is mediated by toll-like receptors (TLR) that detect pathogen-associated molecular patterns (PAMP), mainly in the form of unmethylated CpG dinucleotide sites in viral DNA (Hemmi et al. 2000). TLR2 and TLR9 activation leads to induction of innate immunity through increased production of cytokines and type I interferons (IFNs) (Kawai and Akira 2006, Zhu et al. 2007). IFN secretion shuts down cellular mechanisms of virus replication on auto- and paracrine level, whereas cytokines and chemokines trigger an inflammatory response that recruits innate immune cells to eliminate the virus (Thaci et al. 2011). Neutrophils, natural killer (NK) cells and macrophages help controlling the virus infection by eliminating the infected cells (Hendrickx et al. 2014). Dendritic cells (DCs) and macrophages can also internalize, process and cross-present cellular fragments and proteins in context of major histocompatibility complex (MHC) class I and II, thus acting as professional antigen presenting cells (APCs) (Nayak and Herzog 2010). PAMP-induced maturation of APCs leads to effective presentation of viral antigens to CD4+ helper and CD8+ cytotoxic T-cells (Muruve 2004). While primed CD8+ T-cells can directly kill virus-infected cells, helper CD4+ T-cells provide activation signals for B-cells that produce antibodies against adenoviral proteins (Bradley et al. 2012).

The systemic use of serotype 5 adenovirus is limited by high levels of pre-existing humoral immunity in the general population with up to 90 % seroprevalence in certain geographical locations (Abbink et al. 2007, Barouch et al. 2011). Neutralizing antibodies (NAbs) IgM, IgA, and IgG directed against hexon, penton and fiber proteins can rapidly

opsonize Ad vector upon secondary infection or vector re-administration. NAbs affect virus activity either by sterically limiting cellular association, or by preventing virus uncoating and nuclear entry of viral DNA (Gall et al. 1996, Varghese et al. 2004, Sumida et al. 2005). Fiber pseudotyping using chimeric Ad5/3 virus can partially protect the virus from pre-existing anti-Ad5 antibodies but anti-Ad5/3 NAbs develop after repeated administration of the vector (Sarkioja et al. 2008). However, the presence of NAbs does not seem to hinder the efficacy of virotherapy when Ad is administered intratumorally, at least according to some clinical reports (Tong et al. 2005, Koski et al. 2010).

Wild-type adenovirus can typically cause mild flu, conjunctivitis and infantile gastroenteris due to preferential infection of epithelial cells in eyes and the respiratory and gastrointestinal track (Mautner et al. 1995, Kunz and Ottolini 2010). In contrast, patient data of modified oncolytic adenoviruses have reported grade 1-2 adverse reactions including fever, flu-like symptoms and hematological disturbances, but only few, more disease-related severe adverse events (SAE) such as gastrointestinal problems and thrombocytopenia (Liikanen et al. 2013, Bramante et al. 2014). In general, oncolytic adenoviruses have been considered well-tolerated and dose-limiting toxicities have not been detected (Toth and Wold 2010). However, the efficacy of oncolytic Ad as single-agent modality has left room for improvement, increasing the demand for combinatorial approaches.

1.3.1.3.2 Anti-Tumor Responses

In addition to inducing anti-viral immunity, oncolytic adenovirus can elicit a strong immune response against solid tumors. Active virus-mediated oncolysis leads to release of tumor-associated antigens (TAAs), which are phagocytosed and processed by APCs. These mature APCs, activated by viral danger signals such as PAMPs, effectively cross-prime effector and memory CD8+ T-cells that migrate to the tumor and exert specific lytic activity towards cancer cells (Cerullo et al. 2012, Kaufman et al. 2015). While most approaches rely on inclusion of immunostimulatory transgenes into the viral genome,

also mere backbone Ad can induce anti-tumor immunity. Intratumoral injection of unarmed Ad has led to immunological responses and signs of efficacy both in preclinical (Ruzek et al. 2002, Edukulla et al. 2009, Tuve et al. 2009) and clinical reports (Nokisalmi et al. 2010, Pesonen et al. 2012a). Moreover, efficacy of replication-competent Ad5 in poorly permissive tumor models has been shown to be significantly greater in immunocompetent mice compared to their athymic counterparts (Hallden et al. 2003), highlighting the importance of adaptive anti-tumor immunity as a part of the overall mechanism.

To further boost tumor-specific T-cell responses, oncolytic adenoviruses can be armed with different cytokines and growth factors. Some popular approaches over past years have been the use of immunostimulatory factors such as granulocyte macrophage colony-stimulating factor (GM-CSF) and CD40 ligand (CD40L), both of which aim to enhance the activity and function of APCs and thus indirectly affect priming and activity of anti-tumor T-cells (van Kooten and Banchereau 2000, Arellano and Lonial 2008). In addition, high local concentration of transgene product following Ad replication in tumor and subsequent low systemic exposure has made oncolytic Ad especially attractive in terms of immunotherapy (Bristol et al. 2003). Studies with GM-CSF expressing oncolytic adenovirus have revealed that systemic anti-tumor immunity and memory responses are induced, protecting animals from tumor re-challenge (Cerullo et al. 2010). IL-12 expressing oncolytic Ad was found effective in curing syngeneic pancreatic tumors and inducing anti-tumor immune response (Bortolanza et al. 2009). Finally, oncolytic Ad coding for tumor necrosis factor α (TNF-α) was reported to induce immunogenic cell death and increase local levels of tumor-specific T-cells, resulting in significantly improved efficacy over unarmed virus (Hirvinen et al. 2015).

The immunological characterization of other armed oncolytic adenoviruses have been sparse, as the lack of optimal animal models permissive for adenoviral replication has prevented the complete analysis of mechanism-of-action, especially in terms of oncolysis. In contrast, several reports of non-replicating adenoviruses coding for

cytokines such as IL-12, IL-23, IFN-α, IL-2, TNF-α and CD40L have shown improved treatment efficacy and increase in immune infiltrates compared to unarmed viruses (Addison et al. 1995, Wright et al. 1999, Santodonato et al. 2001, Peter et al. 2002, Raja Gabaglia et al. 2007, Reay et al. 2009, Diaconu et al. 2012), providing proof-of-concept data that could also be extended to replicating, oncolytic platforms. Further vector development and rational use of immunostimulatory transgenes necessitate preclinical evaluation, as many cytokines and growth factors can also induce immune cells associated with tumor immunosuppression and tolerance, possibly leading to less desirable effects (Bronte et al. 1999, Bayne et al. 2012, Boyman and Sprent 2012).

Immune effects of oncolytic immunotherapy using GM-CSF armed, 5/3 chimeric adenovirus has been extensively studied in human patients. GM-CSF is a growth factor involved in stimulation of granulocytes and monocytes, and it can induce both immunosuppressive cell subsets, such as myeloid-derived suppressor cells (MDSC) and M2 macrophages, but also activate immunostimulatory subsets, such as DCs, M1 macrophages and NK cells (Parmiani et al. 2007). In fact, preliminary human data suggests that GM-CSF coding Ad can lead to CD8+ anti-tumor immunity at least in periphery and reduce immunosuppression at tumor level, thus constituting a novel form of *in situ* tumor vaccine (Kanerva et al. 2013, Ranki et al. 2014, Hemminki et al. 2015, Vassilev et al. 2015). No induction or increase in MDSCs or M2 macrophages has been reported to date, indicating that Ad5/3-D25-hGMCSF can manipulate the TME in favor of immune responses rather than tolerance (Pesonen et al. 2015, Vassilev et al. 2015). Presumably the presence of adenovirus, production of GM-CSF and the subsequently secreted cytokines can constitute an optimal cocktail that can polarize the TME towards Th1 phenotype (Shi et al. 2006). However, disturbances in this delicate balance might explain why all patients have not benefited and raise the question whether specific biomarkers could reveal possible responders (Liikanen et al. 2015, Taipale et al. 2015). In addition, oncolytic adenovirus coding for CD40L has shown promising signs of immunological activity in patients (Pesonen et al. 2012b), indicating that transgenes

capable of stimulating adaptive immune responses via APC activation can be effective approach in terms of oncolytic immunotherapy.

1.3.2 Vaccinia Virus

Vaccinia virus (VV) is a prototype poxvirus that has historically been used in vaccination programs against smallpox, another member of the poxvirus family. The family of Poxviridae can be divided into 69 species and 28 genera, of which VV is classified into orthopoxvirus genus. The Western Reserve (WR) strain of VV is a laboratory strain derived from the Wyeth strain through several decades of cultivation and *in vivo* passage in research laboratories (Guse et al. 2011). The WR strain has natural tropism for cancer cells, probably due to a leaky vasculature (McFadden 2005). In addition, VV is highly lytic, capable of spreading through the blood stream and has a large genome which enables insertion of immunostimulatory transgenes, making it an ideal vector for oncolytic immunovirotherapy (McCart et al. 2001, Guo et al. 2005, Kim et al. 2009).

1.3.2.1 Structure and Life Cycle

Infectious vaccinia virus is a double-stranded DNA virus covered by a lipoprotein envelope that structurally resembles the host cell membrane (Upton et al. 2003). The linear genome of VV encodes all the enzymes and proteins necessary for viral replication, as the entire replication cycle takes place in the host cell cytoplasm, outside the nucleus. Some of the viral products have immune evading properties, enabling VV to establish infection in target tissues (Moss 1990, Smith 1993).

Life cycle of vaccinia virus can be divided in early and late phases, first of which starts when VV enters the target cell via membrane fusion, mediated by entry-fusion complex (Carter et al. 2005, Senkevich et al. 2005). Both cellular receptors and the viral determinants essential for VV binding and infection are still unknown, although several attempts to characterize any widely expressed receptors have been made (Eppstein et al.

1985, Lalani et al. 1999, Chahroudi et al. 2005; 2005). After entering the cytoplasm, early stage of transcription is initiated which leads to uncoating of viral particles, modulation of cellular metabolism and replication of viral DNA. This is followed by transcription of intermediate and late genes, which are involved in the production of membrane proteins and structural proteins for new virions (Moss 2012). Most of the intracellular mature viruses (IMV), that serve an important role in inter-host transmission, are released during cell lysis and lack the outer membrane (Sodeik et al. 1993). Alternatively, some IMVs are enwrapped with an additional membrane in Golgi apparatus, actively transported to cell surface and released via direct budding as extracellular enveloped viruses (EEV) (Schmelz et al. 1994). In contrast to IMV particles, EEVs can evade the immune recognition due to a host-derived envelope, facilitating cell-to-cell infection and subsequently leading to systemic VV spread into distant sites (Payne 1980, Smith et al. 2002). The entire life cycle of VV is fast and usually completed within 24 hours from infection (Salzman 1960).

1.3.2.2 Modifications

Vaccinia viruses have a natural tissue tropism for cancer cells, which produce high concentrations of nucleotides needed in viral replication. In addition, leaky blood vessels might facilitate the entry of VV to the tumor site (Thorne et al. 2005). To further enhance safety and tumor-selectivity, VV genome can be genetically engineered. Viral thymidine kinase (TK) is a necessary part of VV replication in normal cells, as low levels of nucleotides are present in non-dividing cells (Buller et al. 1985). In contrast, proliferating cancer cells express high levels of TK and subsequently produce sufficient amounts of deoxyribonucleotides (McKenna et al. 1988). Deletion of TK from the viral genome restricts VV replication to cancer cells overexpressing transcription factor E2F, which upregulates expression of cellular TK (Buller et al. 1985, Shen and Nemunaitis 2005). Similarly, vaccinia growth factor (VGF) genes can be deleted from the viral DNA to reduce replication in normal cells. The secretion of VGF from wild type VV infected cells leads to proliferation of nearby cells by binding to EGFR, which in turn increases

the cellular levels of nucleotides and creates favorable conditions for further VV spread. The deletion of VGF ensures that VV replication only takes place in cancer cells with activated EGFR-Ras pathway (Kirn and Thorne 2009). Finally, double-deleted vaccinia virus (VVdd) featuring both deletions has been developed with reduced pathogenicity and enhanced selectivity (McCart et al. 2001, Haddad et al. 2012), demonstrating potent oncolytic activity and possible utility in cancer immunotherapy (Parviainen et al. 2015).

1.3.2.3 Immune Responses

Although VV has been considered to be an immunogenic virus due to its successful use as a small pox vaccine, it harbors several immunosuppressive features. Following viral entry, type I and II interferons and other inflammatory cytokines are rapidly secreted from host cells, leading to the induction of an anti-viral state (Samuel 1991, Perdiguero and Esteban 2009). As a countermeasure, VV encodes viral proteins such as B18R and B8R, which can bind these cytokines with high affinity and thus neutralize their activity (Smith et al. 2000). Activation of complement can lead to direct lysis of infected cells or to phagocytosis of viral particles by macrophages and neutrophils via opsonization. To counteract this, VV expresses and secretes virus complement proteins (VCPs) which bind complement components, thus preventing activation of complement cascade (Kotwal and Moss 1988). Also antigen cross-presentation may be compromised, as VV has been reported to attenuate APC function despite inducing their maturation (Deng et al. 2006, Yao et al. 2007). Moreover, VV expresses several anti-apoptotic proteins which inhibit elimination of virus-infected cells via induction of apoptosis caspase cascade (Kettle et al. 1997, Taylor et al. 2006).

Both humoral and cellular immunity play a role in anti-VV protection, as individuals with defects in either branches of immunity are unable to control VV infection (Lane et al. 1969). Known envelope proteins of VV can elicit anti-viral NAbs (Galmiche et al. 1999) and preclinical studies have revealed that these NAbs can protect mice from VV infection and disease (Belyakov et al. 2003). In addition, both NK cells and T-cells have

been shown to contribute to resistance to VV infection (Karupiah et al. 1990, Selin et al. 2001, Harrington et al. 2002). CD4+ Th-cell dependent NAb secretion from B-cells has been described critical for VV clearing, also by preventing viral replication (Xu et al. 2004). The role of anti-viral CD8+ T-cells appears to be less significant in primary infection and more important during secondary infection due to formation of immunological memory (Harrington et al. 2002, Xu et al. 2004).

Unarmed, oncolytic vaccinia virus can exert impressive anti-tumor efficacy in transplantable animal models, mainly due to robust oncolysis (Parviainen et al. 2014, Parviainen et al. 2015). First-in-human phase I with double-deleted VV suggested that even unarmed virus can elicit signs of immune cell activation, at least in peripheral blood (Zeh et al. 2015). Instead, the complex nature of human tumors may limit the intratumoral spread of VV and thus reduce the treatment effect. To enhance VV potency as an immunotherapeutic approach, viral replication has been coupled with expression of different immunostimulatory transgenes, which can boost anti-tumor immune responses. B18R-deleted vaccinia virus coding for IFN-β has been reported to induce complete tumor responses and immune-mediated protection against tumor re-challenge in mice (Kirn et al. 2007). GM-CSF is one of the most studied arming approaches in terms of VV immunotherapy with indications of induced anti-tumor immunity both in preclinical models (Parviainen et al. 2015) and in human studies (Mastrangelo et al. 1999). Lastly, CD40L-expressing VVdd was recently introduced but this approach did not seem to provide a significant benefit in anti-tumor efficacy compared to unarmed VV in immunocompetent mice (Parviainen et al. 2014). CD40L has been reported to interfere with VV- and Vesicular Stomatitis Virus (VSV)-based immunovirotherapy by eliciting anti-viral immunity (Ruby et al. 1995, Galivo et al. 2010), possibly explaining the lack of additive effect of the transgene *in vivo*. Moreover, VVdd-CD40L seems to preferentially favor induction of NK cells and MDSCs rather than anti-tumor T-cells (Parviainen et al. 2014), suggesting that the choice of transgene may be critical if prominent CTL responses are desired.

1.4 Adoptive T-Cell Therapy

Immunotherapy using autologous anti-tumor T-cells is potentially a highly effective treatment option for patients with advanced cancer. Adoptive T-cell therapies (ACT) to date include transfer of *ex vivo* expanded tumor-infiltrating lymphocytes (TILs), peripheral blood lymphocytes transduced with high-affinity T-cell receptor (TCR) targeting HLA-restricted tumor antigens, and peripheral blood lymphocytes transduced with chimeric antigen receptor (CAR) targeting antigens on tumor cell surface (Wu et al. 2012). The latter two approaches depend on single specificity of anti-tumor T-cells, whereas personalized TIL therapy has the advantage of targeting broad spectrum of tumor (neo-) antigens. On the other hand, generation of genetically re-engineered anti-tumor T-cells from blood derivates is considered technically more straightforward as lack of resectable tumors or the inability to expand TILs are not limiting factors. Re-targeted tumor-specific T-cells can also potentially be used in allogeneic setting, thus enabling the development of ACT into "off-the-shelf" pharmaceutical product. Taken together, all three approaches demonstrate that cytotoxic tumor-specific T-cells have the potential to eliminate cancer cells and destroy established tumors given favorable circumstances.

1.4.1 Cytotoxic T-Cells in Cancer

Cytotoxic T-cell (CTL) is a lymphocyte that can kill cancer cells, virally infected cells and damaged cells in a TCR specific manner. Recently, a concept of cancer-immunity cycle was proposed in order to explain how tumor antigens can trigger a cytolytic immune response (Chen and Mellman 2013). In this cycle, tumor-associated antigens (which can be either viral proteins, mutated neo-antigens, derepressed embryonic antigens, over-expressed differentiation antigens or normal self-antigens) are released from tumor cells concomitantly with different danger-associated molecular patterns (DAMPs) associated with trauma, cellular stress, hypoxia and depletion of nutrients (such as ATP and HMGB1). Binding of DAMPs to cell surface and intracellular

receptors (including TLRs) induces maturation of professional APCs, especially DCs, which endocytose and process both exogenous and endogenous antigens in TME (Spel et al. 2013, Boone and Lotze 2014).

Following maturation, DCs migrate to secondary lymphoid organs and present the processed antigens to effector CTLs in context of MHC class I (signal 1). CTLs are called CD8+ T-cells because the interaction between MHC I and TCR must be accompanied by a glycoprotein CD8, which acts as a co-receptor that binds to the constant portion of MHC I molecule and keeps T-cell and APC bound closely together during antigen-specific activation. In addition to formation of MHC class I-peptide-TCR complex, CD4+ T helper (Th) licensing via CD40-CD40L interaction is needed to induce costimulatory interaction (signal 2) between CTL and APC (Nesbeth et al. 2010a). Either B7-1 (CD80) or B7-2 (CD86) on mature DC binds to CD28 receptor on the surface of CTL and this engagement enables differentiation, activation and proliferation of T-cells. In the absence of signal 2, T-cells become anergic and can undergo apoptosis as a protective mechanism to prevent formation of autoimmunity. Finally, primed CTLs migrate to TME and infiltrate the tumor bed, where TCR can recognize specific MHC I-peptide complexes on tumor cells and release cytotoxic granules (containing granzyme B and perforins) that mediate direct apoptosis of tumor cells by triggering a cascade of caspases. Presence of TILs has been accepted as an independent prognostic factor in various cancer types including colorectal cancer (Naito et al. 1998), breast cancer (Yoshimoto et al. 1993), ovarian cancer (Sato et al. 2005) and malignant melanoma (Haanen et al. 2006).

1.4.2 T-Cell Therapy Based on Tumor-Infiltrating Lymphocytes

Adoptive T-cell transfer of *ex vivo* expanded TILs in the treatment of metastatic melanoma patients was pioneered by Rosenberg and colleagues, who already in the late 1980's described anti-tumor activity of TILs grown in the presence of "T-cell growth factor", also known as interleukin 2 (IL-2) (Yang and Rosenberg 1988). Autologous

TILs can be expanded from either fragments or enzymatic digests of a resected tumor (Yang and Rosenberg 1988, Dudley 2011). In the initial expansion phase TILs are cultured in growth medium with IL-2 for a 5-week period (Dudley et al. 2003). The intermediate product ("pre-REP" TIL) is then used to generate the final TIL infusion product using a rapid expansion protocol (REP) (Riddell and Greenberg 1990, Dudley et al. 2003). In most cases, a minimum of 50×10^6 T-cells are needed after the pre-REP phase to yield a sufficient cell number for infusion. The historical overall success rate for this has been 60-70 % (Dudley et al. 2003, Dudley et al. 2005, Goff et al. 2010) but current protocols can achieve success rates of 80 % and higher (Besser et al. 2010b, Dudley et al. 2010, Goff et al. 2010). The second expansion phase involves T-cell activation using anti-CD3 antibody and irradiated autologous or allogeneic feeder cells. Two days after initiation of REP, IL-2 is added to induce T-cell proliferation and TILs are expanded for additional 12 days in culture (Dudley and Rosenberg 2003, Dudley et al. 2003). Finally, 1,000- to 2,000-fold expanded TILs are harvested, concentrated and infused intravenously into the patient.

In the past, systemic administration of high dose IL-2 was considered to be an essential part of successful TIL transfer, as it drives TIL survival and expansion *in vivo* (Robbins et al. 2004, Rosenberg and Dudley 2004, Huang et al. 2005). In contrast, recent studies have suggested that intermediate or even low dose of IL-2 might be sufficient (Ellebaek et al. 2012). Concomitant to post-treatment IL-2, the patient undergoes preconditioning regimen prior to TIL infusion. Lymphodepleting chemotherapy (such as cyclophosphamide and fludarabine) is used to eliminate endogenous T-cells including T regulatory cells (Tregs) which can interfere with effector TIL function and persistence (Dudley et al. 2005, Wang et al. 2005, Dudley et al. 2008b). In addition, total-body irradiation (TBI) can be used to further enhance the effect (Dudley et al. 2008b, Rosenberg et al. 2011).

Recently, several novel strategies have been employed to improve the function of autologous TILs. To avoid terminal differentiation, the pre-REP expansion period of

TILs has been reduced to few weeks in the "young TIL" approach (Dudley and Rosenberg 2003, Rosenberg et al. 2008). These minimally cultured TILs have longer telomeres and express higher levels of effector memory markers CD27 and CD28 compared to traditional TIL cultures (Tran et al. 2008). Artificial antigen-presenting cells (aAPC) have been studied in order to reduce costs related to feeder cells, since they can be engineered to express several costimulatory ligands and even secreted or membrane-bound cytokines (Maus et al. 2002, Suhoski et al. 2007). Finally, for enhanced T-cell migration to tumor site, TILs have been transduced with chemokine receptor CXCR2 as its ligands, chemokines CXCL1 (KC) and CXCL8 (IL-8), are produced by tumor cells and tumor-associated stromal cells (Peng et al. 2010).

TIL therapy capitalizes on polyclonal T-cell infiltrates, which are able to recognize multiple tumor-associated antigens (TAA). In fact, flow cytometric screening of TAA-specificities has revealed that only a small fraction of TILs is specific against well-defined melanoma-associated antigens and the rest (over 90 %) of TILs react against unknown antigens (Hadrup et al. 2009, Andersen et al. 2012). Some of these TILs recognize mutated self-proteins (i.e. neo-antigens) (Kvistborg et al. 2012, Robbins et al. 2013b, Cohen et al. 2015) but also other, non-related epitopes derived from cytomegalovirus, Epstein-Barr virus or influenza A virus, emphasizing on the notion that not all TILs are tumor-specific (Andersen et al. 2012, Kvistborg et al. 2012). In addition, only a small proportion of TILs have been reported tumor-reactive and responsible for the most of the tumor cell killing (Kvistborg et al. 2012, Brown et al. 2015), highlighting the need to identify and enrich specific TIL subsets over bystander TILs to enhance therapeutic efficacy.

1.4.3 T-Cell Therapy Based on Genetically Modified T-Cells

Re-programming peripheral T-cells can be achieved by transduction with a retrovirus encoding TAA-specific TCR genes. Engineering blood T-cells enables generation of high numbers of high-affinity CTLs with known specificity for adoptive transfer, unlike

in TIL therapy where T-cell specificities are largely unknown (Hadrup et al. 2009, Andersen et al. 2012). On the other hand, the nature of TCR limits the applicability of a certain TCR construct to only a subset of individuals, as TCR only recognizes a specific peptide in the context of a specific MHC I. Currently only HLA-A0201 has been targeted, which narrows the amount of potential patients to about 30 % of population that express at least one HLA-A0201 allele (Wu et al. 2012). The major limitation with TCR-transduced T-cells, like with TILs, is that they are vulnerable to tumor MHC I downregulation, which can render these T-cells ineffective in recognizing their target cells (Hicklin et al. 1999). In addition, mispairing of introduced TCR α and β chains with endogenous TCR chains is a concern and might lead to reactivity against non-tumor self-antigens (Bendle et al. 2010). Different approaches are currently under investigation to circumvent these problems, including structural modifications of TCRs (Kuball et al. 2007) and knocking down the endogenous TCR (Provasi et al. 2012).

Gene modification of blood T-cells using chimeric antigen receptor (CAR) is an applicable approach in situations where TILs or TCR-modified T-cells are ineffective. Adoptive CAR T-cell (CAR-T) therapy enables usage of non-classical T-cell targets such as cell surface proteins, carbohydrates and glycolipids. As the recognition does not depend on antigen processing and MHC I presentation pathways, the same CAR construct can be used in all patients regardless of HLA type (Sharpe and Mount 2015). The first generation of CAR consisted of a single-chain variable fragment (scFv) linked to transmembrane and cytoplasmic tail of CD3ζ co-receptor (Eshhar et al. 1993) (**Figure 2**). CAR specificity was based on the scFv part, which contains variable domains of heavy and light chains of a monoclonal antibody recognizing tumor antigen (Sadelain et al. 2003). First-generation CAR-Ts were able to induce anti-tumor responses but the lack of co-stimulation in the tumor rapidly led to poor proliferation and anergy of these cells *in vivo* (Heslop 2010). As ζ chain signaling alone was not sufficient to activate CAR-Ts (Brocker and Karjalainen 1995, Ramos and Dotti 2011), second-generation CAR-Ts were designed to include intracellular co-stimulatory molecules such as CD28, CD134 (OX-40) and CD137 (4-1BB) (Hombach et al. 2001, Maher et al. 2002). This two-signal

model for T-cell activation resulted in enhanced anti-tumor activity (Kowolik et al. 2006, Milone et al. 2009) and sparked the development of third-generation CAR-Ts with tandem co-stimulatory endodomains. These modifications further improved persistence, cytokine production and anti-tumor efficacy of CAR T-cells (Finney et al. 2004, Carpenito et al. 2009, Zhong et al. 2010). Recently, a concept of fourth-generation CAR-Ts was introduced in the form of T-cells redirected for universal cytokine-mediated killing (TRUCK) (Chmielewski and Abken 2015). TRUCK is based on secretion of an immunostimulatory cytokine (such as IL-12) from CAR-Ts following interaction with their target cells, which in turn modulates TME and attracts innate immune cells to destroy tumor cells that are not recognized by CAR-T (Chmielewski et al. 2011, Chmielewski et al. 2014). Moreover, IL-12 secretion by CAR-T may eliminate the need of preconditioning regimens usually associated with ACT (Pegram et al. 2012).

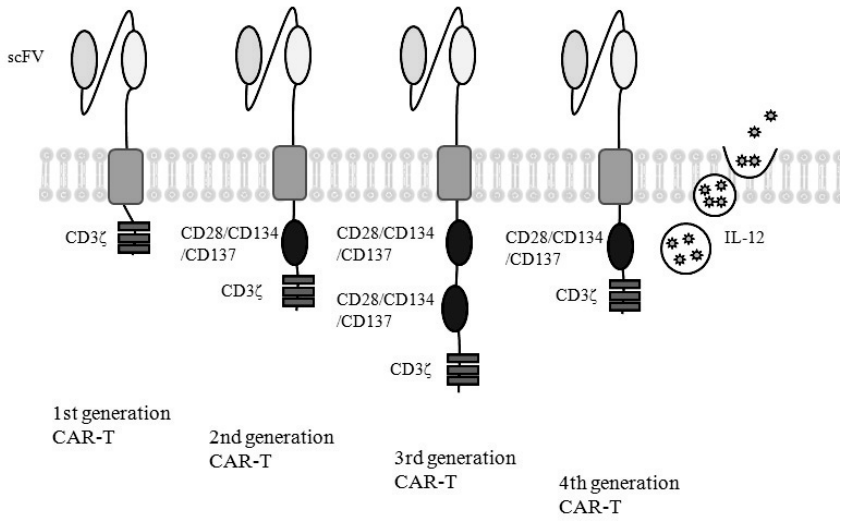

Figure 2. Schematic structure of different generations of CAR-modified T-cells. First generation CAR-T comprised of a single-chain variable fragment (scFv) linked to a transmembrane and cytoplasmic tail of CD3ζ co-receptor. Second and third generation CAR-Ts were designed to include one or two intracellular co-stimulatory molecules such as CD28, CD134 and CD137. Fourth generation CAR-T included all of these elements and was further modified to secrete immunostimulatory cytokines (such as IL-12) into the tumor microenvironment.

The extent of tumor-specific, cell surface antigens may prove to be the main limitation in the development of novel CAR constructs. As TIL and TCR therapies are based recognition of MHC I-peptide complexes, many intracellular antigens have been mapped and characterized over the years (Hinrichs and Restifo 2013). In contrast, before the concept of CAR-T therapies, there has been limited amount of studies on tumor cell surface antigens that could be used as targets for highly specific cancer immunotherapy without on-target off-tumor activity. An ideal target antigen for CAR T-cells would have strong, unique and stable expression on tumor cells. Unfortunately, most cell surface antigens expressed on tumor cells are also expressed on normal tissues, resulting in toxicity and autoimmune manifestations. Furthermore, the exact tissue distribution of many antigens is unknown, which makes prediction of possible SAE difficult. Consequently, inducible Caspase 9 "safety switches" have been designed to allow quick elimination of infused T- cells by administration of a small molecule dimerizer drug in case of adverse events (Di Stasi et al. 2011).

1.4.4 Immune Responses

Several clinical studies have been conducted with T-cells expressing transgenic TCRs or CAR-Ts in the treatment of solid tumors with some reported on-target and off-target toxicities. High-affinity TCRs against different epitopes of melanoma-associated antigen A3 (MAGE-A3) have demonstrated unexpected cross-reactivity against brain or cardiac tissue proteins in two separate trials, resulting in deaths in both cases (Linette et al. 2013, Morgan et al. 2013). TCR targeting carcinoembryonic antigen (CEA) caused transient inflammatory colitis in metastatic colorectal cancer patients due to CEA expression on normal colonic mucosa (Parkhurst et al. 2011). In addition, TCR-modified T-cells specific for melanocyte differentiation antigens gp100 and MART-1 have been causing autoimmune adverse events including skin rash, uveitis and hearing loss (Johnson et al. 2009). First-generation CAR T-cells recognizing carbonic anhydrase IX (CAIX) antigen on renal cancer cells resulted in serious on-target off-tumor hepatotoxicity (Lamers et al. 2006). In another clinical study, third-generation CAR-T against ERBB2 (HER2)

induced the release pro-inflammatory cytokines (a condition known as cytokine release syndrome, CRS), leading to pulmonary toxicity, acute multiorgan failure and death of the patient following T-cell infusion (Morgan et al. 2010). Moreover, multiple doses of mesothelin-specific CAR T-cells created with mRNA-based approach have been reported to induce an anaphylaxis reaction in one patient, probably due to development of IgE antibodies against CAR (Maus et al. 2013).

In theory, as CAR-T modifications include insertions of co-stimulatory molecules, these T-cells might display activity towards their original targets as long as endogenous TCRs are not knocked out. If these TCRs would be targeted against antigens expressed on vital organs, concerns have been raised whether unexpected toxicities could result as "the brakes are off". However, some healthy tissues or cell populations can be killed without causing major complications. CAR-T therapy targeting CD19 can induce massive tumor regression in patients with chronic lymphocytic leukemia (CLL) and acute lymphoblastic leukemia (ALL) (Kochenderfer et al. 2010, Kalos et al. 2011, Porter et al. 2011, Brentjens et al. 2013, Grupp et al. 2013b). The on-target off-tumor activity of CD19 CAR-Ts also leads to depletion of normal B-cells but this can be compensated with immunoglobulin replacement therapy (Maude et al. 2015; 2015). Prolonged B-cell deficiency is not a life-threatening condition and provides a perfect example of an expendable tissue if significant anti-tumor efficacy can be expected.

Anti-tumor effect of ACT might also depend on transferred cells working in cooperation with host immune cells. Short-lived, mRNA engineered CAR-Ts, which might be safer compared to traditional lentivirus-generated CAR-Ts, have been shown to induce *de novo* immune responses in the form of novel anti-tumor antibodies (Beatty et al. 2014). Preclinical studies have concluded that harnessing endogenous immune cells is an effective way to expand tumor-specific T-cell repertoire and eliminate tumor cells that have lost the expression of target antigen following ACT (Nesbeth et al. 2009, Nesbeth et al. 2010b, Spear et al. 2013). Also a few human studies have indicated that adoptive T-cell transfer can broaden the repertoire of endogenous anti-tumor T-cells. Adoptive

TCR therapy targeting MART-1 was reported to increase blood levels of T-cells specific for gp100, tyrosinase and NY-ESO-1 in two melanoma patients with best tumor responses (Ma et al. 2013). Similarly, in another study, one patient treated with MART-1-specific CTL clone experienced a complete response, which was accompanied by expansion of new clonotypes of higher avidity following ACT (Vignard et al. 2005). These case reports provide important clues about the possible mechanism underlying effective T-cell therapies and warrant further studies on the effect of adoptively transferred cells on endogenous immune cell subsets.

High selective pressure by monospecific TCR- and CAR-modified T-cells in the absence of epitope spreading can induce immune evasion, where antigen-negative tumor cell clones achieve growth advantage and contribute to disease relapse (Grupp et al. 2013a, Maude et al. 2014, Sotillo et al. 2015). Formation of immune escape variants could be circumvented by targeting simultaneously at least two different tumor-associated antigens. In B-cell malignancies, a combination of CD19 and CD123 targeted CAR-Ts has resulted in more pronounced efficacy compared to monotherapies in preclinical studies (Ruella et al. 2015). Dual targeted CAR-Ts have been developed and have exerted promising signs of safety and enhanced effector function over monospecific CAR-Ts (Hegde et al. 2013). In addition, a recent study showed that T-cells engineered to express secretable bi-specific T-cell engager (BiTE) specific for CD3 and EphA2 can redirect resident T-cells towards tumor cells (Iwahori et al. 2015). A further improvement of this approach could be a CAR construct targeting one TAA and secreting BiTE targeting a second TAA, enabling a dual T-cell response against the tumor.

In TIL therapy, on-target off-tumor activity should not be an issue, since TILs targeting self-antigens have low-affinity TCRs due to central tolerance (Xing and Hogquist 2012). Moreover, TILs targeting neo-antigens derived from tumor mutations should, by definition, be strictly tumor-specific (Cohen et al. 2015). Still, some TIL treated patients develop autoimmune manifestations such as vitiligo and uveitis, but these autoimmune

reactions do not seem to correlate with objective antitumor responses (Dudley 2005). More importantly, majority of TIL therapy associated toxicities are linked to preconditioning regimens (especially TBI) and systemic administration of high dose IL-2, both of which can cause grade III or IV toxicities and even death (Dudley 2005, Dudley et al. 2008c, Besser et al. 2010c).

1.5 Immunotherapy of Solid Tumors

Effective immunotherapy of solid tumors is based on i) inducing potent and specific anti-tumor immunity and ii) blocking the immunosuppressive counter-responses elicited by solid tumors. Several approaches have included 1) oncolytic viruses that induce immunogenic cell death, release PAMPs and trigger systemic anti-tumor immunity, 2) adoptive T-cell transfer using TILs or genetically re-directed blood lymphocytes to increase the numbers of anti-tumor T-cells, 3) recombinant cytokines to enhance maturation of antigen-presenting cells and activity of cytotoxic T-cells, 4) checkpoint inhibitors to reduce T-cell unresponsiveness and 5) cancer vaccines to induce memory response.

1.5.1 Immune Evasion and Tumor Resistance

Although encouraging clinical signs of efficacy following active and passive immunotherapy have been observed in several tumor types, a substantial number of patients have derived little to no benefit due to immune evasion and resistance mechanisms employed by solid tumors. In terms of immunotherapy, tumor resistance can be divided in intrinsic, naturally acquired and therapy-induced resistance (Kelderman et al. 2014) (**Figure 3**).

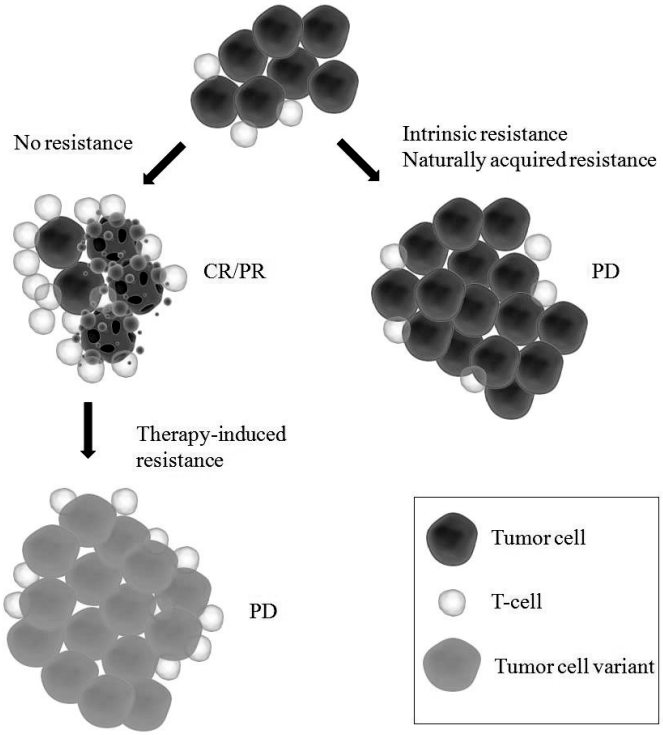

Figure 3. Categories of resistance related to cancer immunotherapy. Intrinsic and naturally acquired resistance to anti-tumor immunity result in progressive disease (PD), whereas patients which initially respond (complete response, CR or partial response, PR) eventually relapse due to therapy-induced rise of resistant tumor cell variants.

Intrinsic resistance refers to a situation where a subset of patients do not respond to treatments due to the lack of anti-tumor immunity and failure to elicit T-cell responses against tumor-associated antigens either on systemic or local level. This applies to immune-compromised patients with HIV infection and patients receiving immunosuppressive medication following organ transplantation, both of which carry an increased risk of virally induced cancers such as Kaposi's sarcoma (Butel 2000). Systemic intrinsic resistance can also be formed when tumors (over-) express self-antigens that are susceptible to peripheral tolerance, leading to low avidity of the available T-cell repertoire (Kvistborg et al. 2013). However, human tumors have the

capacity to express viral-derived or mutated neo-antigens, which are epitopes recognized as foreign by the immune system. Several reports indicate that immune detection of such neo-antigens may hold the key to successful tumor control (Heemskerk et al. 2013, Robbins et al. 2013a, van Rooij et al. 2013, Brown et al. 2014, Champiat et al. 2014, McGranahan et al. 2016). Thus, tumors without viral origin and/or with a low mutational load may be efficient in evading immune recognition due to lack of immunogenic antigens (Alexandrov et al. 2013a, Vogelstein et al. 2013a). Lastly, local intrinsic resistance may occur in the form of hostile tumor microenvironment, which may prevent tumor-infiltration or effector function of anti-tumor T-cells even though systemic responses are induced (Gajewski et al. 2010, Taube et al. 2012, Lutz et al. 2014). Tumor-level expression of PD-L1 and secretion of inhibitory molecules such as TGF-β, IL-10 and IDO preceding T-cell infiltration can have a direct inhibitory effect on T-cell functionality in TME (Geissmann et al. 1999, Steinbrink et al. 1999, Braun et al. 2005). Furthermore, indirect immunosuppression by tolerogenic immature DCs, myeloid-derived suppressor cells (MDSCs), Tregs, tumor-associated macrophages (TAM) and tumor-associated neutrophils (TAN) form a barrier for T-cell mediated tumor destruction (Lutz and Schuler 2002, Harlin et al. 2006, Vukmanovic-Stejic et al. 2006, Strauss et al. 2007, Gabrilovich et al. 2012).

Naturally occurring immune pressure can lead to reduced sensitivity of anti-tumor T-cells in a process called naturally acquired resistance, which is unique to immunotherapy. In this type of situation, signs of ongoing immune response can be detected in periphery and tumor tissue but these patients fail to benefit from immunotherapy. Multiple inhibitory feedback loops in TME can affect T-cell activity, such as expression of checkpoint molecules PD-1, CTLA-4, LAG-3, TIM-3 and BTLA (Pardoll 2012). For example, upon binding to specific TAA, TIL can secrete IFN-γ which in turn can upregulate PD-L1 expression on the surface of tumor cells (Spranger et al. 2013). This leads to interaction between PD-1 on TIL and PD-L1 on tumor cell and triggers T-cell exhaustion (Taube et al. 2012). In addition, naturally occurring immune responses can induce immunoediting of tumors, leading to downregulation of MHC class

I expression on tumor cells (Khong et al. 2004, Pandha et al. 2007) or antigen-negative tumor cell variants (Khong and Restifo 2002, Matsushita et al. 2012a). T-cell dependent immunoselection process has been identified as a key factor in the formation of less immunogenic tumor cell clones evading immune-mediated anti-tumor attack (Shankaran et al. 2001, DuPage et al. 2012, Matsushita et al. 2012b), strongly contributing to naturally acquired resistance.

The aforementioned upregulation of immune checkpoint molecules can also occur following T-cell based immunotherapy, in which case it is defined as therapy-induced resistance. In classical oncology, therapy-induced resistance has usually been associated with cytotoxics and targeted therapies such as BRAF inhibitors (Chapman et al. 2011, Holohan et al. 2013). Treatment with such targeted therapies can result in enrichment of resistant tumor clones due to selection pressure and mutations. Similarly, any immunotherapeutic approach targeting a single TAA can potentially augment tumor immunoediting into antigen-negative and/or less immunogenic tumor cell variants (Sampson et al. 2010). In terms of oncolytic viruses, resistance can be acquired following repeated virus administration via several different mechanisms including interferon signaling (Liikanen et al. 2011a, Liu et al. 2013a), production of neutralizing antibodies (White et al. 2008) and recruitment of anti-viral immune cells (Fulci et al. 2007), resulting in therapy-induced anti-viral immunity in patients naïve to virus prior to therapy.

1.5.2 Clinical Application of Oncolytic Immunotherapies

Both oncolytic viruses and re-directed anti-tumor T-cells can be considered oncolytic immunotherapies, as both utilize viral vectors to specifically target and lyse tumor cells. More importantly, these therapies can also stimulate immunity against both targeted and non-targeted TAAs, potentially resulting in a polyclonal anti-tumor response that may delay or inhibit the onset of immune evasion. In addition, both approaches have shown emerging clinical signs of induced anti-tumor immunity coupled with promising signs

of efficacy. These effects could be further enhanced if tumor resistance could be reduced or circumvented, allowing development of ACT and oncolytic viruses into curative cancer immunotherapies.

1.5.2.1 Oncolytic Viruses

A few unarmed oncolytic adenoviruses have been tested in clinical studies. 5/2 chimeric ONYX-015 resulted in 15-21 % response rate in the treatment of head and neck cancer in phase I-II clinical trials (Ganly et al. 2000, Nemunaitis et al. 2001), but failed to induce observable responses in other tumor types such as pancreatic and ovarian cancer (Vasey et al. 2002, Hamid et al. 2003, Hecht et al. 2003). A highly similar oncolytic adenovirus H101 (Oncorine®) also showed good safety and efficacy in clinical trials (Yu and Fang 2007), and was later approved for the treatment of head and neck cancer in China (Garber 2006). In contrast, most recent approaches in the field of oncolytic virotherapy have relied on inclusion of immunostimulatory transgenes, of which GM-CSF seems to be most commonly used in clinical studies to date. Different oncolytic adenoviruses based on fully serotype 5 or 5/3 chimeric fiber and hGM-CSF arming have shown signs of anti-viral and anti-tumor immune cell activation and disease stabilization in patients treated in personalized treatment program ATAP (Cerullo et al. 2010, Koski et al. 2010, Bramante et al. 2014). Similarly, Ad5/3-D24-hGMCSF (ONCOS-102) therapy resulted in 40 % rate of stable disease (SD) in recently completed phase I (clinicaltrials.gov/ct2/show/results/NCT01598129 on January 5, 2016), and is currently in entering phase I/II trials as mono- and combinatorial therapy.

Other successfully implemented GM-CSF armed viruses in clinical settings include oncolytic vaccinia virus JX-594 (Pexa-Vec) and oncolytic herpes simplex virus (HSV) Talimogene laherparevec (T-VEC, Imlygic®). Initial phase I study of seven melanoma patients treated with JX-594 showed one partial response (PR) and one complete response (CR) (Mastrangelo et al. 1999) and subsequent phase I trials resulted in disease stabilization in 9/10 patients with primary or metastatic liver cancer and in 10/15 patients

with colorectal cancer (Park et al. 2008, Park et al. 2015). As a randomized phase II study with Pexa-Vec in refractory, advanced liver cancer patients failed to meet its primary endpoint of overall survival, upcoming phase III will focus on first-line hepatocellular carcinoma patients (Breitbach 2015).

Out of the several oncolytic viruses studied in clinical trials, T-VEC was the first to receive approval from US Food and Drug Administration (FDA) and European Medicines Agency (EMA) in October 2015 (Ledford 2015). Initial phase I trial showed disease stabilization in three patients with breast cancer or malignant melanoma (Hu et al. 2006). Subsequent phase II study with T-VEC focused on solely on late-stage melanoma, resulting in 8/50 patients experiencing CR, 5/50 experiencing PR and 10/50 experiencing SD (Senzer et al. 2009). Furthermore, induction of systemic host immune responses against non-injected lesions was observed both studies, suggesting that both regional and distant metastases can be targeted (Hu et al. 2006, Senzer et al. 2009, Kaufman et al. 2010). Results of phase III study were recently published and showed that intralesional T-VEC improved the overall response rate from 5.7 % to 26.4 % and durable response rate from 2.1 % to 16.3 % when compared to subcutaneous GM-CSF (Andtbacka et al. 2015). The apparent success of T-VEC paves the way for clinical approval of other oncolytic viruses and highlights their underlying immunostimulatory potential that could be harnessed for vigorous cancer immunotherapy.

1.5.2.2 Adoptive T-Cell Therapies

In clinical studies, autologous TIL transfer in the treatment of metastatic melanoma has resulted in 39 % response rate without lymphodepletion (Dudley 2005, Dudley et al. 2008b) and in 50 % response rate when including cyclophosphamide and fludarabine in the treatment protocol (Dudley et al. 2005, Dudley et al. 2008a, Besser et al. 2010a). Significant enhancement of objective clinical response to 72 % and impressive 40% rate of CR was seen when TBI of 12 Gy was also included (Dudley et al. 2008a, Rosenberg et al. 2011). Notably, these clinical responses came at the cost of significant toxicity,

raising the question of acceptable risk-to-benefit ratio. Besides melanoma, testing of TIL therapy has been limited to few solid tumor types. In a recently published clinical protocol, 3/9 metastatic cervical cancer patients treated with human papillomavirus-targeted TILs experienced objective tumor responses, including two CRs (Stevanovic et al. 2015). Overall response rate of 82 % was seen in ovarian cancer patients without IL-2 administration in early human trials (Aoki et al. 1991), but subsequent trials failed to reach similar results and no clinical responses was observed (Freedman et al. 1994). In renal cell carcinoma, first study with TILs and low-dose IL-2 resulted in overall response rate of 35 % (Figlin et al. 1997). The following randomized phase III trial provided no additional benefit from TILs when compared to IL-2 treatment alone, probably due to significant difficulties in TIL manufacturing (Figlin et al. 1999). More recently, a new clinical trial was started to study the usability of TIL therapy in these and other tumor types such as pancreatic cancer, gastric cancer and hepatocellular carcinoma (Andersen et al. 2015).

In contrast to major success in hematological malignancies (Kochenderfer et al. 2010, Kalos et al. 2011, Porter et al. 2011, Brentjens et al. 2013, Grupp et al. 2013b), the efficacy of CAR T-cells in solid tumors has been somewhat disappointing. CAR-Ts targeting CAIX in metastatic renal cell carcinoma did not result in any objective clinical responses in 11 treated patients (Lamers et al. 2006). Similarly, treatment with CAR-modified T-cells against α-folate receptor did not induce any tumor responses in 14 patients with ovarian cancer (Kershaw et al. 2006). More recently, 3/16 osteosarcoma patients infused with HER2 targeted CAR-Ts showed stable disease (Ahmed et al. 2015). In neuroblastoma trials, 6 patients were treated with anti-L1-CAM CAR T-cells without any clinical responses (Park et al. 2007) but CAR-Ts targeting GD2 led to complete response in 3/11 patients (Louis et al. 2011). Currently, several clinical trials in solid tumor indications are ongoing, including CAR-Ts targeting mesothelin in metastatic mesothelin+ cancers, EGFRvIII in glioma, VEGFR2 in metastatic melanoma and renal cancer, and HER2 in glioblastoma, sarcoma and other HER+ malignancies (Fousek and Ahmed 2015).

Compared to CAR-Ts, TCR-modified T-cells appear to be more efficient in the treatment of solid tumors, possibly due to selection of more amenable cancer types. In a clinical trial using high affinity TCR for CEA in metastatic colorectal cancer, 1/3 treated patients experienced a partial tumor response (Parkhurst et al. 2011). TCR therapy targeting melanocyte antigens gp100 and MART-1 led to treatment benefit in 3/16 and 6/20 melanoma patients, respectively (Johnson et al. 2009). In the previously discussed trial with severe neurological toxicity, MAGE-A3-targeted TCR therapy was reported to induce objective responses in 5/9 patients with one complete response (Morgan et al. 2013). Finally, TCR-transduced T-cells specific for HLA-A2 restricted NY-ESO-1 resulted in tumor responses in 4/6 patients with synovial cell sarcoma and in 5/11 patients with melanoma, two of them having CR (Robbins et al. 2011). The aforementioned results from various clinical studies indicate that ACT can be a powerful approach in the treatment of solid tumors as long as key questions concerning toxicity, optimal target antigens and the need for preconditioning regimens are resolved.

1.5.3 Other Approaches in Cancer Immunotherapy

1.5.3.1 Recombinant Cytokines

Modulation of anti-tumor immune response by using recombinant cytokines is another approach in cancer immunotherapy. The major limitation of recombinant cytokines is their instability and short half-live, necessitating high systemic concentrations for substantial effects to occur, often resulting in toxicities as in the case of IL-2 (Tagawa 2000). Although several recombinant cytokines have been studied in clinical trials, only IFN-α and IL-2 have become established forms of therapy in solid malignancies. IFN-α2b (Intron A®) is used in patients with melanoma, AIDS-related Kaposi's sarcoma and cervical intraepithelial neoplasms (Vacchelli et al. 2014b). IL-2 (Aldesleukin®) is approved for treatment of metastatic melanoma and renal cell cancer and has shown durable complete response rate of 7 % (Atkins 2002, Atkins et al. 2004). However, the associated SAE has limited the use of high-dose IL-2 in the clinics, which has led to

development of IL-2 variants with greater therapeutic index and less toxicity (Shanafelt et al. 2000, Levin et al. 2012). In addition, growth-stimulating cytokines such as GM-CSF (Leukomax®, Mielogen, Leukine®) and granulocyte colony-stimulating factor (G-CSF) (Neupogen®) have been used to reconstitute the immune system after chemotherapy or lymphoablating regimens (Arellano and Lonial 2008), but have not been used as standalone anti-tumor immunotherapeutic agents. Finally, tumor necrosis factor α (TNF-α) has been EMA approved for the treatment of soft-tissue sarcoma and melanoma, administered as isolated limb perfusion in order to limit systemic toxicity (Deroose et al. 2011a, Deroose et al. 2011b). Currently several new clinical studies are ongoing, most of them focusing on rational combinations with recombinant cytokines such as IL-2, IFN-α, IL-7, IL-15 and GM-CSF (Vacchelli et al. 2014b).

1.5.3.2 Monoclonal Antibodies

The clinical utility of monoclonal antibodies (mAbs) was initially limited due to complications associated with administering murine mAbs. These complications included suboptimal ability of murine Abs to co-operate with human immune cells and the induction of host anti-mAb immune responses that elicited rapid clearance of mAbs (Weiner 2015). However, recent advances in genetic engineering techniques have led to generation of chimeric (33 % mouse protein), humanized (5-10 % mouse protein) and fully human mAbs that can mimic the behavior of natural occurring human IgGs (Imai and Takaoka 2006). Mechanistically mAbs can be divided in two categories, immunostimulatory mAbs and tumor-targeting mAbs.

Immunostimulatory mAbs function by specifically binding to and either activating co-stimulatory receptors or inhibiting immunosuppressive receptors (Aranda et al. 2014). Immune checkpoint inhibitor targeting CTLA-4, known as Ipilimumab (Yervoy®), was the first immunostimulatory mAb to get FDA approval in 2011 (Sondak et al. 2011). Since then, antagonistic mAbs targeting PD-1, known as nivolumab (Opdivo®) and pembrolizumab (Keytruda®), have been approved for the treatment of melanoma, RCC

and non-small cell lung cancer (Postow et al. 2015). Also mAbs blocking PD-L1 are currently in phase II and III trials (Planchard et al. 2015, Spira et al. 2015). Although the concept of checkpoint blockade in both cases is similar, CTLA-4 is thought to inhibit T-cells in priming phase (regulating *de novo* immune responses), whereas PD-1 – PD-L1 axis augments T-cell inhibition in effector phase (influencing ongoing T-cell responses).

Several agonistic mAbs are currently under clinical evaluation. Anti-CD40 mAbs capable of inducing tumor cell apoptosis and APC maturation have been studied in phase I trials for the treatment of melanoma, pancreatic ductal adenocarcinoma (PDAc) and other solid tumor types (Aranda et al. 2014, Hassan et al. 2014). The concept of BiTe was recently introduced, having the potential to re-direct T-cells against tumor-associated surface antigens (Nagorsen and Baeuerle 2011). So far only CD19 x CD3 BiTe Blinatumomab (Blincyto®) has received FDA approval for the treatment of B-cell acute lymphoblastic leukemia, whereas phase I trial with EpCam x CD3 BiTe for the treatment of EpCam+ solid tumors is ongoing (Sheridan 2015). Moreover, trifunctional mAb Catumaxomab (Removab®), consisting of EpCam x CD3 antigen binding sites and intact Fc region, has been EMA approved for treatment of malignant ascites (Bokemeyer 2010).

In contrast to immunostimulatory mAbs, tumor-targeting mAbs bind to malignant cells and exert antineoplastic effects by inhibiting signaling pathways necessary for survival and/or proliferation (Vacchelli et al. 2014a). However, treatment with some of these mAbs such as anti-HER2 Trastuzumab (Herceptin®) and anti-EGFR Cetuximab (Erbitux®) can also lead to selective opsonization of malignant cells, resulting in engagement of innate immunity in the form of antibody-depended cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) (Hubert and Amigorena 2012, Rogers et al. 2014). An important realization so far has been the observation that immunoglobulin subclass IgG1 can mediate ADCC but IgG4 cannot (Weiner 2015). This has been taken into account in the development of several novel

immunostimulatory and tumor-targeting mAbs where ADCC is required for full therapeutic effect.

1.5.3.3 Cancer Vaccines

Therapeutic cancer vaccines are designed to eradicate tumor cells through strengthening the patients' own immune responses. Despite extensive clinical testing, the historical response rate of therapeutic vaccines using peptides, whole tumor cells, pox viruses or DCs has remained in 3.8 % (Rosenberg et al. 2004). The only approved cancer vaccine to date is Sipuleucel-T (Provenge®), which is based on *ex vivo* pulsing of peripheral APCs with GM-CSF and prostate antigen PAP. Sipuleucel-T received FDA approval in 2010 for the treatment of asymptomatic metastatic castrate-resistant prostate cancer (Cheever and Higano 2011), as phase III trial of more than 500 patients showed improvement in overall survival from 21.7 months in the control arm to 25.8 months in the vaccine arm (Kantoff et al. 2010). Many other therapeutic vaccines are currently under development or being evaluated in clinical trials, including tumor cell vaccines, protein/peptide vaccines and genetic (DNA, RNA and viral) vaccines (Guo et al. 2013).

The highly immunosuppressive microenvironment in solid tumors can quickly eliminate or neutralize vaccine-elicited T-cells responses (McGray et al. 2014), which may partly explain the lack of anti-tumor activity in previous vaccine trials. Consequently, recently launched clinical trials with cancer vaccines have focused on rational combinations with checkpoint inhibitors and other immunomodulatory agents to counteract regulatory immune mechanisms (Melief et al. 2015). Other major obstacles of cancer vaccinations strategies have been the lack of local inflammatory cues and relatively poor understanding of the optimal way to induce strong and sustained T-cell response against solid tumors. As the understanding of tumor immunology increases, several novel approaches are expected to follow in the upcoming years, including oncolytic viruses as *in situ* cancer vaccines.

2 AIMS OF STUDY

1. To evaluate approaches to enhance anti-tumor efficacy and reduce antiviral immunity in the context of oncolytic virotherapy (I, IV)
2. To study the homing and function of adoptively transferred T-cells following intratumoral injection of immunostimulatory agents (II, III)
3. To identify mechanism-of-action of anti-tumor efficacy induced by combination of adoptive T-cell therapy and oncolytic adenovirus (II)
4. To study the impact of immunostimulatory cytokines on tumor microenvironment in the context of adoptive T-cell therapy (III)

3 MATERIALS AND METHODS

3.1 Cell Lines

In all the studies, oncolytic adenovirus production was done in human lung adenocarcinoma cell line A549 and titering was performed with human E1-transformed embryonic kidney cell line HEK293. Production and titering of replication-deficient adenoviruses was done in HEK293 cells. In study I, vaccinia virus production was done in A549 cells and titering in African green monkey kidney epithelial cell line Vero. In studies I and IV, ovarian carcinoma Skov3-Luc cells were used for bioluminescence imaging as these cells stably express transgenic firefly luciferace. In studies I, II and III, *in vivo* experiments were conducted with murine melanoma B16.OVA cells, which express chicken ovalbumin (OVA) as surrogate tumor antigen. All cell lines used in the studies are summarized in **Table 1**.

Table 1. Cell lines used in the studies.

Cell line	Description	Species	Source	Used in study
A549	lung adenocarcinoma	human	ATCC	I, II, III, IV
HEK293	transformed embryonic kidney cells	human	Microbix	I, II, III, IV
Vero	kidney epithelial cells	African green monkey	ATCC	I
786-O	renal carcinoma	human	ATCC	I

Skov3-Luc	ovarian carcinoma	human	provided by Dr. Negrin (Stanford Medical School, Stanford, CA)	I, IV
B16.OVA	melanoma	mouse	provided by Prof. Vile (Mayo Clinic, MN)	I, II, III
B16.F10	melanoma	mouse	ATCC	II

All cell lines were maintained in either RPMI 1640 or DMEM containing 10 % fetal calf serum (FCS), 1× L-glutamine and 1× penicillin/streptomycin. In addition, 10 % G-418 was added into the growth medium of B16.OVA cells.

3.2 Patient Tumor Explant Tissues

Fresh ovarian cancer tissue samples were obtained from Women's Hospital of Helsinki University Central Hospital with informed consent and ethical committee permission (373/E6/2003). Primary tumor tissue from patients undergoing surgery was placed in a chilled 50 ml Falcon tube containing DMEM supplemented with 10 % FCS, L-glutamine and penicillin/streptomycin and transported to BSL-2 laboratory for further processing.

3.3 Adenoviruses

The replication-deficient adenoviruses used in studies I and II were E1- and E3-deleted adenoviruses with firefly luciferase transgene inserted into the deleted E1 region under cytomegalovirus (CMV) promoter. In study I, neutralizing antibody assay was done with

Ad5/3-Luc, a chimeric serotype 5 adenovirus modified with serotype 3 knob. In study II, pre-immunization of mice was performed with Ad5-Luc, which is fully serotype 5 adenovirus.

The replication-competent adenoviruses used in studies I, II and IV were all 5/3-fiber chimeric, oncolytic adenoviruses harboring a 24 base-pair deletion (D24) in the retinoblastoma (Rb) binding region of E1A. In study I, Ad5/3-D24-TK/GFP was used for fluorescence microscopy as the virus contains a transgene encoding green fluorescent protein (GFP). In study II, experiments were conducted with Ad5/3-D24 and Ad5/3-D24-hGMCSF. Although the latter contains a transgene, it was considered to be unarmed as human granulocyte macrophage colony-stimulating factor (hGMCSF) is not biologically active in murine cells (Shanafelt et al. 1991).

Production and characterization of replication-deficient and replication-competent adenoviruses are described in the original publications and references. Briefly, adenoviruses were amplified in A549 or HEK293 cells, purified on double cesium chloride gradients and titered by spectrophotometry (for VP/ml) and by standard tissue culture infectious dose 50 ($TCID_{50}$) assay (for PFU/ml). Furthermore, presence of transgenes and other genetic modifications and the absence of wild-type adenovirus were confirmed with polymerase chain reaction (PCR). All adenoviruses used in the studies are summarized in **Table 2**.

Table 2. Adenoviruses used in the studies.

Virus	Transgene	Used in study	Reference
Ad5/3-Luc	luciferase	I	(Kanerva et al. 2002)
Ad5-Luc	luciferase	II	(Kanerva et al. 2002)
Ad5/3-D24	-	II, IV	(Kanerva et al. 2003)

Ad5/3-D24-TK/GFP	green fluorescent protein	I, IV	(Raki et al. 2007)
Ad5/3-D24-hGMCSF	human granulocyte macrophage colony-stimulating factor	II	(Koski et al. 2010)

3.4 Vaccinia Viruses

The vaccinia viruses used in study I were of Western Reserve strain with total deletion in the vaccinia growth factor (VGF) and partial deletion in thymidine kinase (TK) gene. Production and characterization of these replication-competent, double deleted vaccinia viruses (VVdd) are described in references (Table 3). Briefly, vaccinia viruses were amplified in A549 cells, purified on sucrose cushion and titered by plaque assay (for PFU/ml) in Vero cells. Presence of transgenes was confirmed PCR and fluorescence microscope. All vaccinia viruses used in the studies are summarized in **Table 3**.

Table 3. Vaccinia viruses used in the studies.

Virus	Transgene	Used in study	Reference
VVdd-Luc	luciferase	I	(Guse et al. 2010)
VVdd-tdTomato	tdTomato	I	(Parviainen et al. 2014)

3.5 *In Vitro* Studies

3.5.1 Cytotoxicity Assays

To study cell killing efficacy of oncolytic adenovirus on murine B16.OVA cells (study I), B16.OVA cells were plated in 96-well plates (1×10^4 cells/well) and infected in triplicates with different concentrations of Ad5/3-D24-TK/GFP in 2% RPMI. Progress of infection was monitored daily and cell viability was assessed 13 and 22 days post-

infection by CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega) according to manufacturer's instructions.

To study synergistic cell killing of oncolytic adenovirus and oncolytic vaccinia virus (study I), A549 or 786-O cells were seeded in 96-well plates (5×10^4 cells/well) and infected in six replicates with different multiplicity of infection (MOI) of Ad5/3-D24-TK/GFP and VVdd-tdTomato. Cell viability was assessed 72 hours post-infection by CellTiter 96 Aqueous One Solution Cell Proliferation Assay per manufacturer's instructions.

3.5.2 Electron Microscopy

Skov3-Luc cells were infected with Ad5/3-D24-TK/GFP (100 PFU/cell) and VVdd-tdTomato (10 PFU/cell) and sorted for GFP+ TdTomato+ double positive cells 24 hours post-infection. Co-infected cells were gently scraped of the culture dish and fixed immediately with 2 % glutaraldehyde. After storing the samples overnight in +4ºC, cells were dehydrated and embedded in LX-112 resin. Finally, the cells were sectioned, mounted on electron microscope (EM) grids and analyzed under JEOL 1400 Transmission EM.

3.5.3 111In-oxine Cell Labeling

For *in vitro* cell viability assay, 1×10^5 OT-I T-cells were labeled with 0-3.70 Bq 111In-oxine (half-life of 2.83 days) per cell for 15-20 min in room temperature. Radiolabeled OT-I cells were washed twice with growth medium and placed in a 24-well plate wells in lymphocyte media supplemented with anti-CD3e (Abcam) and mIL-2 (R&D Systems). Cells were monitored for 7 days and finally cell viability and proliferation were assessed by Trypan Blue staining. The optimal dose for *in vivo* studies was determined as a dose that didn't induce significant cell death but inhibited lymphocyte

proliferation, so that migration into tumors was the only measured parameter in SPECT/CT imaging.

3.6 *In Vivo* Studies

All *in vivo* studies were carried out in accordance with the Act on the Protection of Animals Used for Scientific or Educational Purposes (497/2013) and the Government Decree on the Protection of Animals Used for Scientific or Educational Purposes (564/2013). Animal protocols were reviewed and approved by the National Animal Experiment Board (Eläinkoelautakunta ELLA) of the Regional State Administrative Agency of Southern Finland (ESAVI/4621/04.10.03/2012).

4-7 week old mice were ordered from Harlan Laboratories, Jackson Laboratories or Taconic. Mice were housed in individually ventilated cages in BSL2 level facility and the health status of the mice was examined daily. Intratumoral injections (I, II, III) and *in vivo* imaging (I, II, IV) were performed under isoflurane gas anesthesia. All efforts were made to minimize suffering and mice were euthanized if their general condition deteriorated or if one of the two tumor diameters reached 18 mm.

3.6.1 Animal Models

3.6.1.1 Immunodeficient Mouse Models

In study I, nude Naval Medical Research Institute (NMRI) mice were implanted subcutaneously with 3×10^6 human A549 cells into both flanks. When tumors reached injectable size, xenografts on the right flank were treated intratumorally with either 50 µl PBS, 1×10^8 PFU Ad5/3-D24 or 1×10^7 PFU VVdd-tdtTomato. After 48 hours, mice were treated intravenously with either 1×10^8 PFU Ad5/3-D24 or 1×10^7 PFU VVdd-tdTomato. After another 48 hours, tumors and livers were harvested for plaque assay, TCID50 and qPCR.

Also in study I, nude NMRI mice were implanted subcutaneously with 2.5×10^5 murine B16.OVA cells into right flank. Tumors of injectable size were treated intratumorally with either 50 µl PBS or 1×10^{10} VP Ad5/3-D24 or 1×10^8 PFU VVdd-tdTomato. After 3 days, mice were treated intratumorally with either 50 µl PBS or 1×10^{10} VP Ad5/3-D24 or 1×10^8 PFU VVdd-tdTomato. Growth of subcutaneous tumors was calculated using formula length×width^2×0.52.

In study I and IV, female severe combined immunodeficiency (SCID) mice were implanted intraperitoneally with 3×10^6 human Skov3-Luc cells. In study I, mice were treated intraperitoneally either with 100 µl PBS or 1×10^9 VP Ad5/3-D24. After 48 hours, mice were treated intraperitoneally with either 100 µl PBS or 1×10^8 PFU VVdd-Tomato. The virus treatments were continued weekly for total of four weeks. In study IV, mice were treated intraperitoneally with either 100 µl PBS or ruxolitinib (LC Laboratories). After minimum of 2 hours, mice were treated intraperitoneally either with 100 µl PBS or 1×10^9 VP Ad5/3-D24. Virus treatments were continued once a week and ruxolitinib three times a week. In both studies, growth of intraperitoneal tumors was measured twice a week by IVIS imaging system.

3.6.1.2 Immunocompetent Mouse Models

In studies I-III, immunocompetent female C57BL/6 mice were implanted subcutaneously with 2.5×10^5 murine B16.OVA cells into right flank. Tumors usually became injectable (>3 mm in diameter) on day 10 post-implantation.

In study I, mice were primed intratumorally with either 50 µl PBS or 1×10^{10} VP Ad5/3-D24 or 1×10^8 PFU VVdd-tdTomato. To compensate for the lack of adenovirus replication in the mouse model system, mice were re-injected with 1×10^{10} VP Ad5/3-D24 the following day. Six days post-treatment one set of mice was euthanized and tumors were harvested for plaque assay and TCID50. To study the effect of boosting, the virus regimen was repeated on another set of primed mice using intratumoral

administration of PBS, Ad5/3-D24 or VVdd-tdTomato. Tumor growth was followed for 12-14 days, after which tumors were collected for flow cytometry and serum for neutralizing antibody assay.

In study II, mice were treated intratumorally with 50 µl PBS or 1×10^9 VP Ad5/3-D24-hGMCSF on six consecutive days. On the first day of virus treatment, mice were also adoptively transferred with 5×10^5 or 2×10^6 CD8a-enriched T-cells isolated from spleens and lymph nodes of C57BL/6-Tg(TcraTcrb)1100Mjb/J (OT-I) mice. Tumor growth was followed for 14 days, after which tumors and tumor-draining lymph nodes were collected for flow cytometry and spleens for interferon-γ ELISPOT. For B16.F10 challenge experiment, tumor-naïve or virus/T-cells combination treated, B16.OVA-bearing mice were implanted subcutaneously with 2.5×10^5 murine B16.F10 cells into left flank on day 13 post-transfer. Emergence or growth of B16.F10 tumors was followed for 14 days.

In study III, mice were either left non-injected or injected intratumorally with 50 µl PBS or recombinant cytokines five times a week for two weeks. On the first day of intratumoral treatments, mice also received intraperitoneally an adoptive transfer of 2×10^6 CD8a-enriched OT-I T-cells (in 100 µl plain RPMI). Tumor growth was followed for 14 days, after which tumors were collected for cytokine analysis and flow cytometry.

3.6.2 Isolation and Expansion of T-Cells

Ovalbumin-specific, TCR transgenic OT-I mice recognize OVA residues 257-264 (SIINFEKL) in the context of H-2Kb. For studies with adoptive transfer of T-cells, spleen and lymph nodes were harvested from OT-I mouse and minced in 10 % RPMI using a scalpel. Cells were passed through a 70 µm sterile filter and centrifuged at 1100 rpm for 4 min, after which red blood cells were lysed with Ammonium-Chloride-Potassium (ACK) lysis buffer. Remaining immune cells were washed twice with 10 % RPMI and resuspended in lymphocyte medium (RPMI 1640, 10 % FBS, 20 mM L-Glutamine, 1× Pen/Strep solution, 15 mM HEPES, 50 µM 2-mercaptoethanol, 1 mM Na

pyruvate) supplemented with 160 ng/ml recombinant murine IL-2 (R&D Systems) and 300 ng/ml soluble anti-mouse CD3e antibody clone 145-2C11 (Abcam).

Cells were grown for two days, after which cytotoxic CD8+ T-cells were enriched using mouse CD8a+ T-cell Isolation Kitt II and LS columns (Miltenyi Biotec) per manufacturer's recommendations. Non-target cells (i.e. T helper cells, B cells, NK cells, dendritic cells, macrophages, granulocytes, endothelial cells and erythroid cells) were magnetically labeled with biotin-conjugated antibodies and anti-biotin MicroBeads, allowing depletion of non-CD8a+ cells. The flow-through, representing the enriched CD8+ T-cells, was collected and resuspended in lymphocyte medium with mIL-2 and anti-CD3e. T-cells were expanded in numbers for five additional days, harvested and adoptively transferred into recipient B16.OVA-bearing mice.

3.6.3 Recombinant Cytokines

Carrier-free murine cytokines without BSA (**Table 4**) were thawed, reconstituted in PBS at a stock concentration of 100 µg/ml and stored in aliquots at -80ºC. Right before intratumoral administration, one aliquot was thawed, diluted in PBS and kept on ice until use.

Table 4. Recombinant murine cytokines used in the study.

Group	Dose/Mouse/Day (µg)	Dose/Mouse/Day (U)	Commercial supplier
GM-CSF	1	N/A	Invitrogen
IL-2	0.3	3 000	Invitrogen
IFN-α2	0.3	3 000	eBioscience
IFN-γ	1.75	10 000	eBioscience
TNF-α	0.5	N/A	R&D Systems

*N/A = not assessed

3.6.4 Ruxolitinib

To analyze the effect of antiviral signaling on tumor resistance, SCID mice bearing intraperitoneal Skov3-Luc tumors were treated with Janus kinase 1 and 2 inhibitor ruxolitinib. Ruxolitinib phosphate salt (LC Laboratories) was reconstituted in PBS containing 1 % bovine serum albumin (BSA) and injected intraperitoneally (0.4 mg in 100 µl PBS) into mice on days 0, 2, 4, 7 and 9 after starting virus treatments.

3.6.5 NK Cell Depletion

To deplete NK cells, C57BL/6 mice were treated intraperitoneally with anti-asialo GM1 antibody (Wako Chemicals). 35.7 µl of undiluted antibody was given per mouse for total of 4 times before and after B16.OVA tumor implantation (on days -3, 0, 7 and 15). Tumor growth was followed for 14 days, after which spleens were collected for flow cytometry to confirm NK-depletion.

3.6.6 Pre-Immunization

To study the effect of pre-existing anti-Ad immunity, a set of mice was immunized by two intramuscular injections of replication-deficient Ad5-Luc1 (1×10^7 VP in 20 µl PBS). Three weeks later, virus-naïve and pre-immunized B16.OVA-bearing mice were treated intraperitoneally with 2×10^6 CD8a-enriched T-cells on day 0 and intratumorally with 50 µl PBS or 1×10^9 VP Ad5/3-D24-hGMCSF on six consecutive days. Tumor growth was followed for 14 days, after which spleens were collected for flow cytometry.

3.6.7 Bioluminescence Imaging

SCID mice bearing Skov3-Luc xenografts were imaged twice per week with IVIS (Xenogen). Each mouse received 3 mg D-Luciferin (Synchem) in 100 µl PBS intraperitoneally and was anaesthetized with isoflurane. Mice were imaged 8 min later

for 10 sec and relative tumor burden was quantified by IVIS signal intensity (photons/second/cm^2/steradian).

3.6.8 SPECT/CT Imaging

For *in vivo* nanoSPECT/CT imaging, CD8a+ enriched and expanded OT-I cells were suspended in saline and injected into intraperitoneal cavity of recipient mice (with a dose of 4.6 ± 1.3 MBq per animal). Adenovirus- and PBS-treated mice were imaged with a preclinical four-headed gamma camera with integrated CT system (Bioscan Inc) on days 1, 4 and 7 post-transfer. Mice were anaesthetized with isoflurane during the scan. CT images were acquired with 55 kVp X-ray tube voltage and 500 ms exposure time and SPECT images in 16 projections and 230 s per gantry position. The amount of migrated OT-I cells in tumors was quantified as percentage of activity in tumor from the total activity in the whole body divided by the tumor volume (mm^3). Radioactive signal in the tumors was further corroborated by *ex vivo* measurement using gamma counter Wizard 3 (Perkin Elmer) on day 7 post-transfer (study end-point).

3.7 *Ex Vivo* Studies

3.7.1 Infection of Primary Surgical Patient Tissues

Under aseptic conditions, tumor tissue was cut into small pieces (~2 mm^3) using a scalpel and each piece was placed in a 24-well plate well containing 0.5 ml 10 % DMEM. These tumor bits were infected with 1×10^8 PFU Ad5/3-D24-TK/GFP and/or 1×10^7 PFU VVdd-tdTomato per slice. Infected tumor tissue was followed under a fluorescence microscope for 7 days, after which samples were collected for quantification of infectious virus (PFU/g tumor).

3.7.2 Quantification of Viral DNA

In study I and II, murine tumors were collected and digested overnight with proteinase K in tissue lysis buffer ATL at 500 rpm +56^{0}C. Total DNA was extracted using the QIAamp DNA Mini Kit (Qiagen) per manufacturer's instructions. Quantitative PCR targeting the adenoviral E4 gene, vaccinia virus terminal VGF region, human β-actin or mouse β-actin was performed as described earlier (Kanerva et al. 2002, Vähä-Koskela et al. 2015). Primers and probes used are summarized in **Table 5**.

Table 5. Primers and probes used in virus qPCR.

Virus	Name	Sequence (5´-3´)
Adenovirus	FWD	GGAGTGCGCCGAGACAAC
	REV	ACTACGTCCGGCGTTCCAT
	probe	TGGCATGACACTACGACCAACACGATCT
Vaccinia virus	FWD	GATGATGCAACTCTATCATGTA
	REV	GTATAATTATCAAAATACAAGACGTC
	probe	AGTGCTTGGTATAAGGAG
Human β-actin	FWD	TCACCCACACTGTGCCCATCT
	REV	GTGAGGATCTTCATGAGGTAGTCAGTC
	probe	ATGCCCTCCCCCATGCCATCCTGCGT
Mouse β-actin	FWD	CGAGCGGTTCCGATGC
	REV	TGGATGCCACAGGATTCCAT
	probe	AGGCTCTTTTCCAGCCTTCCTTCTTGG

3.7.3 Quantitation of Infectious Virus

Virus from *ex vivo* infected patient tumor bits was rescued by homogenization (using Tissue Master 125 rotor from Omni International) and three freeze-thaw cycles. Adenovirus was isolated by filtering the part of the lysed samples twice through 0.2 µm

filter (Whattman) and centrifugating at 6000 rpm +4^{0}C for 4 min. $TCID_{50}$ assay was performed with filtered samples to quantify infectious adenovirus (PFU/ml). In addition, plaque assay was performed with non-filtered part of the lysed samples to quantify infectious vaccinia virus (PFU/ml).

3.7.4 Neutralizing Antibody Titers

In study I, neutralizing antibodies (NAbs) for adenovirus and vaccinia virus were measured from serum samples of virus-treated mice on day 12 post-treatment. To inactivate the complement system, serum samples were incubated at +56ºC for 90 min and serial dilutions were prepared in plain DMEM. Diluted samples and VVdd-Luc (0.1 PFU/cell) or Ad5/3-Luc (100 VP/cell) were mixed for 30 min in room temperature on a shaker. A549 cells on 96-well plate (1×10^4 cells/well) were washed with plain DMEM and treated in triplicates with serum+virus mix. After 1 h incubation, fresh 10 % DMEM was added per well and cells were incubated ON at +37ºC. Finally, growth medium was removed and cells were lysed with Reporter Lysis Buffer (Promega) and a single freeze-thaw cycle. Bioluminescence was measured with Luciferase Assay System using TopCount luminometer (PerkinElmer).

3.7.5 Cytokine and Chemokine Analysis

For multiplex analysis of murine cytokines and chemokines, 10-100 mg of tumor tissue was collected, snap-frozen in dry ice and stored at -80ºC. Tumors were homogenized with Tissue Master 125 rotor in ice-cold PBS supplemented with 0.1 % BSA and protease inhibitor cocktail (Sigma-Aldrich). Homogenized samples were centrifuged at 2000×*g* +4^{0}C for 10 min and the supernatant was added on pre-wet 96-well filter plate containing Mixed Capture Beads (CBA Flex Set, BD). The plate was shaken for 5 min at 500 rpm and incubated for 1 hour at RT (in foil). Next, PE Detection Reagent was added, plate was shaken for 5 min at 500 rpm and incubated for 1 hour at RT (in foil). Vacuum manifold was applied to aspirate the liquid from wells and the beads were

resuspended in Wash Buffer by shaking the plate for 5 min at 500 rpm. Finally, samples were run with BD FACSArray bioanalyzer or BD Accuri C6 flow cytometer and analyzed using FCAP Array software (BD).

3.7.6 Enzyme-Linked ImmunoSpot (ELISPOT) Assay

For detection of IFN-γ secretion, 1×10^5 live splenocytes from virus- or mock-treated mice were plated into pre-washed and serum-blocked 96-well ELISpot plate. 400 ng of HAdV-5 Penton peptide pool, TRP-2 peptide SVYDFFWL, gp100 peptide KVPRNQDWL or ovalbumin peptide SIINFEKL was added per well and incubated for 45 hours at +37°C (in foil). Cells were removed and the plate was washed 5 times with PBS containing 0.5 % FCS. Detection antibody R4-6A2-biotin was added and incubated for 2 hours at RT. Plate was washed again 5 times and incubated in Streptavidin-ALP for 1 hour at RT. Finally, BCIP/NBT-plus substrate solution was added and the plate was incubated for 20 min (until spots emerged). Color development was stopped by extensive washing with tap water, the plate was left to dry and the number of spots/well was quantified by AID ELISpot Reader System (Aid Autoimmun Diagnostika).

3.7.7 Flow Cytometry

3.7.7.1 Tissue Processing

In studies I, II and III, solid tissues (spleens, tumor-draining lymph nodes and B16.OVA tumors) were minced in 10 % RPMI using a scalpel, treated with ACK buffer to lyse red blood cells and passed through a 70 μm sterile filter to create single-cell suspensions. In some experiments, tumor tissue was incubated in 10 % RPMI supplemented with collagenase type P and benzonase-nuclease for 1-2 hours at 37ºC. After processing, cells were either analyzed immediately or frozen in -80ºC for later analysis.

3.7.7.2 Staining of Surface Markers

In studies I, II and II, approximately 1-2×10^6 cells were allocated per well in a round-well 96-well plate. Cells were washed with FACS stain buffer containing fetal bovine serum and ≤0.09 % sodium and centrifuged at 500×*g* +4^{0}C for 5 min. For pentamer staining, cells were resuspended in stain buffer and incubated with Pro5 MHC Pentamer (10 µl/well) at RT for 20 min (in foil). Cells were washed again and resuspended in antibody cocktail containing stain buffer and optimal amounts of fluorochrome conjugated antibodies. After incubation on ice for 30 min (in foil), cells were washed twice, resuspended in stain buffer and analyzed by flow cytometry (BD FACSAria or BD Accuri C6), counting at least 100,000 events per sample.

3.7.7.3 Staining of Intracellular Markers

In studies II and III, single-cell suspensions of tumors were stimulated for 6 h with 1× Cell Stimulation Cocktail (eBioscience) containing phorbol myristate acetate (PMA) and ionomycin in the presence of brefeldin A. After stimulation, cells were first stained for cell surface antigens, washed and fixed with IC Fixation Buffer (eBioscience) for 20-60 min in RT (in foil). After centrifugation at 400×*g* RT for 5 min, cells were permeabilized twice by resuspending in 1× Permeabilization Buffer (eBioscience) and centrifugating at 400×*g* RT for 5 min. For detection of intracellular antigens, the fixed and permeabilized cells were resuspended in antibody cocktail containing Permeabilization Buffer and optimal amounts of fluorochrome conjugated antibodies. After incubation at RT for 60 min (in foil), cells were washed twice, resuspended in stain buffer and analyzed on BD Accuri C6 flow cytometer, counting at least 100,000 events per sample.

3.7.7.4 Antibodies

All the antibodies and pentamers for flow cytometry are summarized in **Table 6**.

Table 6. Antibodies used in the studies.

Antibody	Host species	Commercial supplier	Catalogue number
CD8b-FITC	rat	eBioscience	11-0083-85
Foxp3-APC	rat	eBioscience	17-5773-82
CD25-PE	rat	eBioscience	12-0251-82
CD19-PE	rat	eBioscience	12-0193-82
H-2Kb-PE	mouse	eBioscience	12-5958-82
H-2Kb-SIINFEKL-PeCy7	mouse	eBioscience	25-5743-80
CD8a-APC	rat	eBioscience	17-0081-82
CD45-APC	rat	eBioscience	17-0451-82
CTLA-4-PE	armenian hamster	eBioscience	12-1522-81
PD-1-PeCy7	armenian hamster	eBioscience	25-9985-80
NK1.1-FITC	mouse	eBioscience	11-5941-81
F4/80-APC	rat	eBioscience	17-4801-82
CD44-FITC	rat	eBioscience	11-0441-81
CD62L-PE	rat	eBioscience	12-0621-81
CD69-PeCy7	armenian hamster	eBioscience	25-0691-82
IFN-γ-APC	rat	eBioscience	17-7311-82
CD31-FITC	rat	eBioscience	11-0311-82
gp38-PE	golden syrian hamster	eBioscience	12-5381-82
CD8a-PerCP-Cy5.5	rat	eBioscience	45-0081-82
CD4-PerCP.Cy5.5	rat	BD	550954
CD3-PeCy7	rat	BD	560591
CD11c-FITC	armenian hamster	BD	553801
Gr-1-FITC	rat	BD	553127
Ly6G-PE	rat	BD	551461
CD11b-PerCP-Cy5.5	rat	BD	550993

Ly6C-APC	rat	BD	560595
CD86-PE	rat	BD	553692
CD3-APC	armenian hamster	BD	553066
CCR7-PerCP-Cy5.5	rat	BD	560812
CD124-PE	rat	BD	552509
CD206-FITC	rat	Biolegend	141704
TIM-3-APC	rat	Biolegend	119705
SIINFEKL-pentamer-APC	-	Proimmune	F093-4B
KVPRNQDWL-pentamer-APC	-	Proimmune	F1333-4A
SVYDFFVWL-pentamer-APC	-	Proimmune	F185-4A-E

3.8 Statistics

Statistical analyses were performed with MedCalc (MedCalc Software), SPSS version 21 (SPSS IBM) and GraphPad Prism 6 (GraphPad Software Inc.). Comparisons between two groups were done using unpaired, two-tailed Student's t-test. Comparisons between multiple groups were done using one-way ANOVA followed by Tukey's post-hoc test. Area-under-curve (AUC) analysis was used for IVIS imaging data and repeated measures ANOVA for log-transformed tumor volume data. Differences were considered statistically significant when p-value was less than 0.05.

4 RESULTS AND DISCUSSION

4.1 Presence of One Oncolytic Virus Does Not Preclude the Infection by Another Virus in Heterologous Virotherapy (I)

Oncolytic viruses can be used as an effective immunotherapeutic approach due to their inherent immunostimulatory capacity which can render tumors susceptible to recognition by the host immune system. At the same time, induction of anti-viral immunity is a problem especially in terms of multiple virus injections, which can lead to anti-viral rather than anti-tumor responses. In vaccine studies, this unfavorable anti-vector response has been circumvented by switching to another, immunologically distinct virus vector coding for the same target antigen. Similar prime-boost approach was recently taken in a preclinical study, where heterologous virotherapy with oncolytic adenovirus Ad5-wt and Lister strain of vaccinia virus demonstrated strong CD3+ T-cell-dependent therapeutic efficacy in hamster models (Tysome et al. 2012). Unfortunately, efficient replication of both oncolytic viruses is unlikely to occur in clinical situations, as human tumors are more complex in terms of heterogeneity, tumor stroma and innate immune responses. We set out to study how limited replication capacity of one of the two viruses, in this case oncolytic adenovirus, would affect the efficacy and anti-viral responses in heterologous adeno-poxvirus combination setting. The hypothesis was that virus-triggered inflammation could offset the lack of replication (Hallden et al. 2003) and that the combination therefore could still be efficacious.

First, we assessed how co-infection of human cancer cell lines and primary tumor tissues affects replication of oncolytic viruses Ad5/3-D24-TK-GFP and VVdd-tdTomato. In spite of saturating doses of both viruses (100 PFU/cell and 10 PFU/cell, respectively), only 10 % of human A549 lung adenocarcinoma cells were found to be GFP+ tdTomato+ double positive (Figure 1a, Study I). Nevertheless, mature virus particles were detected in double positive Skov3-Luc cells by electron microscopy, indicating that co-infection is possible but not preferred (Figure 1c, Study I). Also when primary surgical tumor

samples were co-infected, viruses preferentially occupied individual infection regions but persisted similarly despite heterologous infection (Figure 1e-f, Study I).

To reveal the level of possible systemic virus-virus interference, subcutaneous A549 tumors were established in flanks of nude mice and treated intratumorally with PBS or the first virus. Two days after intratumoral administration, the mice were treated intravenously with PBS or the second virus, and functional titers of both viruses were determined from the tumor. Interestingly, vaccinia virus infection was found from PBS-, Ad- and non-injected tumors (determined by plaque assay and qPCR). In constrast, adenoviral genomes were detected with similar pattern when analyzed by qPCR, but infectious adenovirus was found only from PBS- or VV-injected tumors (Figure 2 and Supplementary Figure 3, Study I), suggesting that physical manipulation of the tumor tissue may promote entry of systematically delivered adenovirus. In conclusion, vaccinia virus seems to be more suitable for systemic administration, especially in the case of priming virus-naïve tumors in adeno-poxvirus therapy.

4.2 Adeno-Vaccinia Virus Combination Therapy Results in Tumor Growth Suppression Even if Adenovirus Replication Is Inhibited (I)

Next, we wanted to study the effect of combination therapy in different mouse models exhibiting either acquired anti-adenoviral resistance (Skov3-Luc) or poor susceptibility to adenoviral replication (B16.OVA). It has previously been shown that SCID mice bearing intraperitoneal Skov3-Luc xenografts can become adenovirus-refractory after repeated virus administration (Liikanen et al, 2011), and similar effect was seen in our experiment (Figure 3a, Study I). Conversely, injection of oncolytic vaccinia virus two days after each adenovirus treatment significantly slowed down the tumor growth and delayed the induction of acquired resistance (Figure 3a, Study I). Of note, eventually the combination treated Skov3-Luc tumors became resilient even to vaccinia virus (day 28 post-infection).

Immunocompetent hamsters are ideal models to study the adenoviral replication and oncolysis, especially of Ad5-based vectors (Diaconu et al. 2010), which is why it is not surprising that Ad-VV combination therapy was highly effective in these animals (Tysome et al. 2012). In order to study optimal treatment regimens of heterologous prime-boost viruses when a tumor is intrinsically semi- to non-permissive for adenoviral replication (as shown in Supplementary Figure 4, Study I), we turned to B16.OVA mouse model. Immunocompetent C57BL/6 mice bearing B16.OVA tumors were treated intratumorally with the first virus (prime) and six days later with the second virus (boost) (Supplementary Figure 5, Study I). Interestingly, groups of mice receiving vaccinia virus prime (VV-Ad, VV-VV) showed statistically significant suppression of tumor growth compared to mice with Ad prime (Ad-VV, Ad-Ad) (Figure 4a, Study I).

4.3 Anti-Viral Immunity and Virus Titers in Mice Treated with Heterologous Prime-Boost Regimen (I)

As expected, homologous Ad-Ad treatment resulted in robust anti-viral immunity (measured by neutralizing antibodies) and reduced adenovirus titers in tumors (Figure 4b and d, Study I). By contrast, Ad-VV prime-boost regimen resulted in low NAb titers and high Ad and VV titers (Figure 4b-d, Study I), whereas VV-Ad regimen reduced Ad and VV levels compared to single treated tumors. This reduction of both virus titers was accompanied by significant increase of NK cells in VV-Ad treated tumors (Figure 5b, Study I), suggesting possible presence of antiviral NK cells. Furthermore, prominent infiltration of CD3+ CD8+ T-cells was detected in all virus-treated tumors compared to PBS-controls (Figure 5a, Study I). Depletion experiments revealed that both of these immune cell subsets proved to be dispensable for the oncolytic efficacy of VV (Figure 5c-d, Study I), thus confirming viral replication and oncolysis to be the main mechanism for anti-tumor activity.

In summary, by contrast to the results of Tysome et al (2012), where the best regimen in virus-permissive, immunocompetent hamsters was Ad prime and VV boost, we found

that restricted replication of the priming virus may affect both i) the efficacy of the combination therapy and ii) determine which heterologous virus regimen would be optimal. In our hands, VV prime seemed to give the best results in terms of overall efficacy, with the heterologous VV-Ad prime-boost only trending toward better tumor growth control compared to homologous VV therapy (p=0.0755). Finally, these results speak for the importance of tumor lysis, in the case for the oncolytic capacity of the virus during priming, which would be expected to release more tumor-associated antigens for antigen cross-presentation, and thereby lead to a greater CD8+ T-cell response compared with priming without lysis. Moreover, such prime-boost regimens could benefit from using oncolytic adenovirus with D24 modification and 5/3 chimeric fiber for enhancing safety and infectivity in human cells compared to wild type Ad5. This small alteration might further enhance virus-induced anti-tumor immune responses in heterologous adeno-pox virotherapy, regardless of the lack of viral replication.

4.4 Oncolytic Adenovirus Improves the Anti-Tumor Efficacy of Adoptive T-Cell Therapy in A Poorly Permissive Model (II)

Adoptive T-cell transfer represents a promising immunotherapeutic approach for treating cancer using *ex vivo* expanded tumor-specific T-cells, derived from either tumor-infiltrating lymphocytes (TILs) or genetically re-directed peripheral blood T-cells. Several pre-clinical and clinical studies have provided proof-of-concept results but poor to modest response rates in advanced solid tumors, probably due to T-cell hypofunction and tumor-induced immunosuppression (Gilham et al. 2012, Moon et al. 2014). So far, the biggest success stories using transgenic T-cells have been accomplished in the treatment of CD19-expressing hematological malignancies using CAR T-cells (Kalos et al. 2011, Grupp et al. 2013b), highlighting the need to develop novel approaches to achieve similar efficacy in solid tumors.

Tumor microenvironment plays a critical role in the outcome of several different cancer therapies (Tsai et al. 2014), especially in the case of immunotherapies based on the

activity of anti-tumor T-cells. TME is a complex network of cancer cells, stromal cells and tumor-infiltrating immune cells, which can be immunosuppressive, immunostimulatory or even display both characteristics depending on the interaction with other immune cells and resulting cytokine milieu in the tumor. Over the course of tumor development, immunosuppressive immune cell populations are preferred as part of the immune evasion tactic that tumors employ to escape from recognition and destruction by the host immune system. We hypothesized that the intrinsic immunostimulatory capacity of oncolytic adenovirus could be utilized to increase tumor immunogenicity, alter cytokine content and change the immune cell balance towards anti-tumor rather than pro-tumor responses.

To assess whether multiple intratumoral injections of adenovirus could be used to mimic infection caused by viral replication, subcutaneous B16.OVA melanoma tumors were treated on six consecutive days with 1×10^9 VP Ad5/3-fiber chimeric adenovirus. Significant suppression of tumor growth was observed (Figure 1a, Study II) with concomitant increase in tumor-specific T-cells (Figure 1e-f, Study II), implicating that adenovirus can induce anti-tumor response despite absence of viral replication. This data is in line with previous studies reporting that adenovirus infection in the tumor can induce anti-tumor activity (Ruzek et al. 2002, Tuve et al. 2009). In addition, antiviral immunity was evoked as copy number of virus genomes in tumors declined over time (Figure 1b, Study II). Similar results in terms of anti-tumor and antiviral effects were obtained in Study I with 10-fold higher adenovirus doses (Figure 4a-b, Study I).

Next, we studied if this adenovirus-mediated increase in tumor immunogenicity would improve the efficacy of adoptive OT-I T-cell transfer. With identical virus-injection regimen as before, significant improvement in B16.OVA tumor growth control was observed both with 5×10^5 and 2×10^6 OT-I T-cells (Figure 2a-b, Study II). Neither lower nor higher T-cell dose alone was able to suppress tumor growth, confirming clinical observations of poor efficacy of T-cell therapy used as a single agent in the treatment of solid, immunosuppressive tumors.

4.5 Antiviral T-Cell Immunity Does Not Reduce Efficacy of the Combination Therapy (II)

Most humans develop anti-adenoviral immunity early in life (Lenaerts et al. 2008), which might affect the efficacy of oncolytic adenoviruses in treatment of human patients. We wanted to investigate the role of pre-existing immunity by pre-immunizing a group of mice twice with Ad5-based vector intramuscularly three weeks prior to intratumoral treatments. Despite induction of antiviral T-cells (Figure 2d, Study II), the tumor growth curves of pre-immunized and virus-naïve mice completely overlapped (Figure 2c, Study II), suggesting that at least in mice pre-existing antiviral T-cells do not hinder the efficacy of combination therapy. Nevertheless, mimicking this type of human situation in mice is challenging due to obvious differences in tropism, replication capacity and encounter frequency of the virus. To overcome pre-existing humoral anti-viral immune responses, alternating the serotype (Mastrangeli et al. 1996) or using capsid modified viruses such as Ad5/3-D24 (Sarkioja et al. 2008) may prove useful. A further approach used in immunovirotherapy is using fully serotype 3 (Ad3) oncolytic adenoviruses which might be especially useful in case of high anti-Ad5 neutralizing antibodies (Hemminki et al. 2011).

4.6 Strong Anti-Tumor Response Following Adenovirus and T-Cell Therapy Is Due To Increase in Endogenous Melanoma-Specific TILs (II)

To evaluate possible immunological factors behind the rigorous therapeutic effect, tumor-infiltrating lymphocyte populations were analyzed. Surprisingly, tumor-trafficking of transferred OT-I T-cells was not enhanced by adenovirus injections (Figure 2e-f, Study II), despite the observation that adenovirus infection can upregulate intratumoral expression of IFN-γ –inducible chemokines (Supplementary Figure 1, Study II). Instead, we observed a significant increase in tumor-infiltration of endogenous CD8+ T-cells targeting melanoma-associated antigens TRP-2 and gp100 following

adenovirus/T-cell combination therapy (Figure 3, Study II). Similar results were obtained from ELISPOT analysis using splenocytes derived from combination treated mice (Figure 4b-c, Study II). In addition, both tumors and tumor-draining lymph nodes (tdLN) of Ad/T-cell treated mice contained higher levels of CD86+ antigen-presenting cells (APCs) and fibroblastic reticular cells (FRCs) than those of control mice, indicating adenovirus-triggered enhancement in antigen cross-presentation (Figure 6 and 7, Study II).

As differences in T-cell responses and in antigen presentation seemed to be the most prominent among the immune cell subtypes analyzed (Figure 3 and Supplementary Figure 4, Study II), we wanted to study whether T-cell activation status was changed following virotherapy. Interestingly, virus-treated mice had increased levels of CD8+ CD69+ CD25+ TILs on day 14 post-transfer compared to PBS-treated controls and significantly less TILs positive for anergy marker TIM-3 than non-injected mice (Figure 5, Study II). Furthermore, adenovirus-treated tumors contained elevated levels of IFN-γ expressing CD8+ TILs compared to control tumors (Figure 4a, Study II), indicating that local immunosuppression was overcome. Taken together, combining adenovirus injections with adoptive T-cell therapy may present a feasible way to enhance activation of anti-tumor immune cells in order to treat highly immunosuppressive solid tumors.

4.7 Systemic Anti-Tumor Efficacy Is Augmented By the Combination Therapy with Adenovirus and Adoptive T-Cell Transfer (II)

In addition to increased levels of endogenous tumor-specific T-cells, a marked reduction in B16.OVA metastasis was seen in tumor draining lymph nodes of combination treated mice on day 14 post-transfer (Figure 4d, Study II). We took this as a promising sign of virus-induced anti-tumor immunity and wanted to study whether these treated mice could reject parental B16.F10 tumor cells not expressing the target antigen OVA. Following B16.F10 challenge, 67 % of mice treated with virus/T-cell combination rejected the tumor challenge, whereas only 10 % of treatment-naïve control mice were

B16.F10-free on day 14 post-implantation (Figure 4e, Study II). This strongly suggests that the combination treatment can induce systemic, endogenous anti-tumor immunity and decrease peripheral tolerance of anti-tumor T-cells.

In conclusion, intratumoral administration of adenovirus can cause a cancer vaccine -like effect by acting as an adjuvant and inducing responses against several naturally occurring tumor epitopes. This type of expansion in T-cell repertoire has been reported previously from several immunotherapy studies targeting a single, well-defined TAA (Lally et al. 2001, Butterfield et al. 2003, Lurquin et al. 2005, Carrasco et al. 2008). It might even prove to be crucial for the clinical success of immunotherapy, since tumors are highly adaptive and a selective pressure by monoclonal T-cell response may easily lead to antigen-negative tumor cell variants (Khong and Restifo 2002, Matsushita et al. 2012a). Moreover, OVA can be considered as a model for mutated tumor-associated (neo-) antigens, which represent an appealing target for CARs and high-avidity TCRs (Hacohen et al. 2013, Heemskerk et al. 2013) in order to avoid off-tumor toxicity and autoimmunity. As neo-antigens are gaining ground in the field of cancer immunotherapy, future studies will show whether targeting neo-antigens can also expand the endogenous T-cell repertoire as suggested here.

4.8 Intratumoral Administration of Recombinant Cytokines Enable Efficient T-Cell Therapy (III)

Unfavorable ratios between different immune cells displaying effector or suppressor properties in TME often limit the efficacy of T-cell based therapies in solid tumors (Kunert et al. 2013). As several recombinant cytokines have been approved for human use for different indications (Vacchelli et al. 2014b), we decided to study whether direct administration of IFN-α2, IFN-γ, IL-2, TNF-α or GM-CSF into tumors would affect the cellular composition of TME and the activity of TCR-transgenic anti-tumor T-cells after adoptive transfer.

Using the previously established model (Study II), mice bearing B16.OVA tumors were treated intraperitoneally with 2×10^6 CD8a+ OT-I cells and intratumorally with different doses of recombinant cytokines (Table 4). On day 14 post-transfer, four out of five cytokines (IFN-α2, IFN-γ, IL-2, TNF-α) combined with T-cells induced significant tumor suppression compared to control groups (Figure 1a and Supplementary Figure 2, Study III). Interestingly, intratumorally administered recombinant GM-CSF exerted growth-stimulatory effect and resulted in increased tumor volumes compared to PBS control group. Similar pro-tumor effect of GM-CSF has been previously described (Bronte et al. 1999, Obermueller et al. 2004, Bayne et al. 2012, Pylayeva-Gupta et al. 2012). However, the dosing and timing of GM-CSF administration seems to be critical, as GM-CSF has been used previously with success as a recombinant cytokine (Geynisman et al. 2013) and as a virus-vectored transgene (Cerullo et al. 2010, Andtbacka et al. 2015).

4.9 Recombinant Cytokine Therapy Leads to Alteration of the TIL profile (III)

To dissect the possible factors underlining the improved efficacy of ACT, we analyzed the phenotype and specificity of T-cells infiltrating the cytokine-treated tumors by flow cytometry. In line with the previous study with adenovirus (Study II), none of the recombinant cytokines were capable of increasing the tumor-levels of transferred OT-I T-cells (Figure 2b-c, Study III). Instead, tumors treated with GM-CSF, IFN-α and IL-2 contained significantly more endogenous CD8+ TILs than control tumors (Figure 5a, Study III) and some of these cells were targeting melanoma-associated antigens TRP-2 and gp100, especially in the case of INF-γ and IL-2 (Supplementary Figure 3e-f). Moreover, intratumoral administration of IFN-γ increased the levels of central memory T-cells (T_{CM}) in tumors, whereas intratumoral IL-2 promoted TIL differentiation into effector memory T-cells (T_{EM}) (Figure 5b, Study III). These examples underline the capacity of cytokine therapy in modifying the T-cell profile of tumors and suggest that recombinant cytokines can induce repertoire expansion of anti-tumor T-cells, although

the extent of the response may be less prominent than seen with adenovirus (Figure 3, Study II).

4.10 *In Situ* Cytokine Therapy Modifies the Cellular Composition of Tumor Microenvironment (III)

Different myeloid- and lymphoid-lineage cells comprise a major part of TME, and the balance between such anti-tumor and pro-tumor immune cells can determine the outcome of adoptive T-cell therapy (Kunert et al. 2013). Hence, we analyzed immune infiltrates of cytokine-treated tumors and found that IFN-α2 and IL-2 favor the proliferation of intratumoral NK cells (Figure 4b, Study III), whereas IFN-γ leads to increased tumor-accumulation of CD11b+ F4/80+ macrophages (Study 4c, Figure III). In contrast, GM-CSF injections skewed polarization of myeloid cell subsets into immunosuppressive M2 macrophages and monocytic MDSCs (Figure 4d and f, Study III), which may explain the poor anti-tumor efficacy of GM-CSF in this study. Moreover, GM-CSF seemed to increase intratumoral levels of tolerogenic DCs, characterized by the lack of co-stimulatory molecule CD86 (Figure 3, Study III). On the other hand, IL-2 administration augmented maturation of these intratumoral CD11c+ dendritic cells (Figure 3, Study III), but also polarized CD4+ TILs into Treg phenotype (Supplementary Figure 3d, Study III). These results suggest that the same cytokine can simultaneously induce both anti-tumor and immunosuppressive responses, which in the case of IL-2 is well-documented (Boyman and Sprent 2012). As the anti-tumor effect of tumor-specific T-cells rely strongly on the cell composition of the TME, detailed characterization of therapy-induced immunosuppressive cell subsets may prove to be crucial in terms of overall efficacy and mechanism.

4.11 Local Administration of Immunostimulatory Cytokines Induces T-Cell Activation and Reduces T-Cell Exhaustion (III)

As recombinant cytokines were found to influence intratumoral myeloid cell populations that can either induce peripheral tolerance or activate anti-tumor T-cells (Gajewski et al. 2013), we studied the activation and exhaustion status of TILs following cytokine therapy. Interestingly, IFN-α2 and IL-2 injected tumors contained higher numbers of activated CD69+ IFN-γ+ CD8+ TILs than control tumors (Figure 5c, Study III), indicating that local cytokine therapy either increased tumor-infiltration of activated T-cells or enhanced activity of pre-existing TILs. Notably, analysis of T-cell exhaustion markers CTLA-4 and PD-1 revealed that *in situ* cytokine treatment reduced the level of TIL exhaustion over time (Figure 6 and Supplementary Figure 4, Study III). This downregulation of CTLA-4 and PD-1 was observed only in the groups with significant anti-tumor efficacy (IFN-α2, IFN-γ and IL-2), whereas control (non-injected and PBS) treated and GM-CSF treated mice exhibited high, constitutive expression of exhaustion markers on CD8+ TILs (Figure 6, Study III).

In summary, these results indicate that carefully selected immunomodulatory cytokine can counteract tumor-associated immunosuppression and T-cell hypofunction, thus enabling effective T-cell therapy in solid tumors. Moreover, the mode of action of such selective immunomodulation can be multi-factorial and include enhanced activity and/or accumulation of several immune cell subsets (Supplementary Figure 5, Study III) that engage the immune system to kill tumor cells, leading to growth suppression or even to complete destruction of the established tumor.

4.12 Interferon Type I Does Not Inhibit Infection and Spread of Oncolytic Adenovirus *In Vitro* (IV)

As antiviral signaling has previously been shown mediate tumor resistance to oncolytic viruses (Liikanen et al. 2011b, Liu et al. 2013b, Ruotsalainen et al. 2015), we examined

whether treatment with small-molecule inhibitor ruxolitinib could improve the efficacy of oncolytic adenovirus in virus-resistant ovarian carcinoma model. Ruxolitinib is known to inhibit Janus kinase 1 (JAK 1), an essential part of IFN-I signaling (Schindler et al. 2007), and has been shown to enhance replication of interferon-sensitive oncolytic VSV *in vitro* (Escobar-Zarate et al. 2013).

To study the direct effect of IFN-exposure on tumor cell protection, Skov3-Luc cells were pre-treated with human IFN-β and/or ruxolitinib and infected two hours later with Ad5/3-D24 or VSV-D51 at 1 PFU/cell. Six days post-infection, viability of Skov3-Luc cells was assessed by Coomassie Blue staining. Interestingly, treatment with IFN type I had no inhibitory effect on progressive infection and oncolysis of Ad5/3-D24 (Figure 1, Study IV). Similar results were obtained with cytokines IFN-γ and TNF-α (Figure 3b, Study I). In contrast, IFN I-sensitive VSV-D51 was completely halted by IFN-β and even low doses of ruxolitinib could fully reverse the antiviral effect, as reported by previous the study (Escobar-Zarate et al. 2013). The inability of soluble antiviral factors to limit the spread of adenovirus was further corroborated in another *in vitro* experiment, where filtered supernatant from adenovirus-infected splenocytes had no inhibiting effect on Ad5/3-D24-TK-GFP infection rate in Skov3-Luc cells (Figure 2, Study IV). The complete lack of adenoviral resistance *in vitro* suggests that mere exposure to soluble interferon or other anti-viral cytokines is not enough to halt replication of oncolytic adenovirus in Skov3-Luc cells and thus do not account for antiviral resistance of the model.

4.13 JAK1/2 Inhibitor Ruxolitinib Can Increase Tumor Control of Oncolytic Adenovirus *In Vivo* (IV)

To analyze if ruxolitinib has an impact on antiviral resistance *in vivo*, we employed the same virus-resistant intraperitoneal Skov3-Luc xenograft model as reported previously (Liikanen et al. 2011b). In contrast to *in vitro* results, ruxolitinib was able to significantly improve the anti-tumor efficacy of oncolytic Ad5/3-D24 in SCID mice during treatment

(Figure 3, Study IV). This is in line with previous observations, where Skov3-Luc tumors were able to resist adenovirus-mediated oncolysis *in vivo* but lost this ability *ex vivo* when tumor stroma was removed (Liikanen et al. 2011b).

The tantalizing difference in adenoviral cell killing capacity *in vivo* and *in vitro* strongly point towards importance of cell-to-cell contact and the presence of stromal or immune cells in the maintenance of anti-viral resistance. Interestingly, in our hands Skov3-Luc tumors started growing again 12 days after last ruxolitinib injection, suggesting that virus-resistance was slowly re-acquired via recovery of some particular anti-viral (immune) cell subset. Despite being immunodeficient by definition, SCID mice have residual immunity such as NK cells (Dewan et al. 2005). The role of NK cells in terms of oncolytic viruses may be complex, since NK cells can exhibit both antiviral (Biron et al. 1999) and anti-tumor properties (Cheng et al. 2013). A recent clinical study described that ruxolitinib impairs NK cell function in patients treated for myeloproliferative neoplasm and this defect is associated with increased virus infection rates (Schonberg et al. 2015a). It is plausible that the additive effect of ruxolitinib on the efficacy of oncolytic adenovirotherapy *in vivo* actually relates to impaired function of anti-adenoviral NK cells. In addition, induction and persistence of antiviral potency of tumor microenvironment may require cell-to-cell contact between antiviral immune (NK) cells and tumor cells, since direct exposure to soluble factors (such as recombinant IFN-β or supernatant from infected splenocytes) alone is unable to inhibit the spread of oncolytic adenovirus.

Tumor infiltration of anti-viral NK cells might also explain the results of Liikanen et al (2011), who showed preliminary evidence of tumor stroma having a role in maintaining antiviral resistance. Out of several pathways analyzed, upregulation of IL-10 signaling pathway and increase in interferon-induced MxA was found in virus-resistant Skov3.ip tumors (Liikanen et al. 2011b). This is especially interesting in the terms of NK cell activation, as IL-10 can upregulate MxA expression in NK cells (Mocellin et al. 2004) and could therefore account for some of the MxA positive subpopulations in Skov3.ip

tumors. Consequently, immune cell composition of tumor microenvironment may be a critical factor in terms of successful adenovirotherapy, as low level of MxA in pre-treatment tumor biopsies has been associated with a trend towards improved overall survival following oncolytic adenovirotherapy in cancer patients (Taipale et al. 2015).

5 SUMMARY AND CONCLUSIONS

The field of oncolytic viruses has experienced a paradigm shift over the last 5-10 years: previously oncolytic viruses were thought to act primarily through viral replication and the subsequent lysis of cancer cells; nowadays the dogma has transitioned towards a concept of therapeutic *in situ* cancer vaccine, where oncolysis leads to release of tumor-associated antigens and virus infection results in danger signals that can be efficiently recognized by host immune cells. Realization of the huge immunostimulatory potential of oncolytic viruses has since resulted in several translational approaches that combine viruses with standard chemotherapy (Cerullo et al. 2011, Liikanen et al. 2013), immune checkpoint blockade (Puzanov et al. 2013) and other cancer therapies (Turnbull et al. 2015).

Repeated administration of the same oncolytic vector suffers from naturally acquired or therapy-induced anti-viral resistance that that may hamper the viral spread and replication. In order to reduce immune responses against the vector, different viruses can be alternated. Such heterologous prime-boost setting has been utilized with various viruses, such as Ad and VSV (Bridle et al. 2009, Bridle et al. 2010), VSV and Maraba virus (Pol et al. 2014), Ad and Semliki Forest virus (SFV) (Näslund et al. 2007), VV and SFV (Vähä-Koskela et al. 2013) and Ad and VV (Tysome et al. 2012). In study I, we examined the latter combination in a situation where replication of one of the viruses was basally or progressively limited, reflecting a possible situation in advanced human tumors. In this setting, we found no conclusive evidence of superiority of one priming virus over another. Instead, it became evident that replication capacity of adenovirus as a prime is important and may enable the immunity-mediated anti-tumor responses observed in the previous report (Tysome et al. 2012). Alternating the vector between Ad prime and VV boost also resulted in delayed induction of therapy-induced resistance, which was associated with improved control of tumor growth. In contrast, vaccinia prime, especially VV-Ad regimen, seemed to induce anti-viral NK cells which resulted in reduced titers of both viruses but did not notably reduce therapeutic efficacy. These

examples underline the complexity of anti-viral immunity and indicate that two distinct viruses elicit immunological consequences both locally and systematically, which, depending on the context, may affect treatment efficacy of oncolytic virotherapy.

Adoptive T-cell therapy can result in impressive clinical results when it's combined with different pre- and post-conditioning regimens (Dudley et al. 2005, Dudley et al. 2008a, Besser et al. 2010a), most of which aim to reduce tumor-level immunotolerance and T-cell hypofunction. However, significant toxicities have been associated with these regimes due to their systemic and non-specific effect which also affects normal tissues (Dudley 2005, Dudley et al. 2008c, Besser et al. 2010c). In study II, we examined whether oncolytic adenovirus could augment efficacy of ACT without concomitant conditioning. Interestingly, intratumorally administered adenovirus was able to reverse tumor immunosuppression and restore activity of TILs, concurrently with inducing an endogenous T-cell response against well-characterized melanoma-associated antigens. This also translated into therapeutic effect, as primary tumors seized growing and majority of the mice were able to reject the challenge of OVA-negative tumor. Induction of such systemic polyclonal T-cell response may be a requisite for successful cancer immunotherapy, as it reduces the risk of tumor immune evasion via targeting multiple epitopes simultaneously.

A critical factor of successful immunotherapy in solid tumors is the tumor microenvironment, which can greatly contribute to hyporesponsiveness of T-cells infiltrating the tumor bed (Kunert et al. 2013). In study III, we analyzed whether cellular composition of tumor microenvironment could be modulated in favor of ACT. By administrating recombinant cytokines daily, we were able to show that selective immunomodulation can increase the anti-tumor efficacy of adoptively transferred T-cells. This increase in T-cell activity was accompanied by changes in quantity and function of effector cells and suppressor cells, tipping the scale towards anti-tumor responses. However, in spite of apparent benefit to anti-tumor efficacy, the poor pharmacokinetics may limit the usability of recombinant cytokines in this setting.

Instead, oncolytic viruses offer an advantageous way to ensure sustained and local expression of the desired transgenes. Several human studies have been performed with non-replicating adenoviruses coding for cytokines such as IL-2, IFN-α and IFN-γ (Dummer et al. 2008, Sterman et al. 2011, Khammari et al. 2015), showing that vector-mediated cytokine delivery is viable concept. Moreover, preclinical data support the use of oncolytic Ad platform to deliver cytokines in the TME in order to improve T-cell therapy (Nishio et al. 2014, Yan et al. 2015).

Importantly, inclusion of cytokine-armed oncolytic adenoviruses in ACT regimens could decrease the need to eliminate other lymphocytes competing with infused TILs for homeostatic γ chain cytokines (such as IL-7 and IL-15) (Klebanoff et al. 2005). Continued production of cytokines could notably increase the levels of intratumoral cytokines compared to endogenous secretion, making lymphodepleting chemotherapy redundant. Moreover, systemic IL-2 administration could be replaced by local virus-vectored production. Would high intratumoral levels of IL-2 be sufficient to induce durable responses depends on the question which one, anergy or lack of proliferation, is the bigger obstacle hindering the current success of ACT in solid tumors. If the transferred T-cells only need re-activation locally after infiltrating the tumor bed, intratumoral IL-2 production may be sufficient. However, if prominent T-cell proliferation and sustained persistence is desired, low-dose systemic IL-2 may be required since clonal expansion of effector T-cells takes place mainly in the secondary lymphoid organs, necessitating IL-2 presence also in the periphery.

Anti-viral NK cells might be of a special concern in the case of IL-2 encoding oncolytic adenoviruses, as IL-2 is a pleiotropic cytokine that affects several immune cell subsets, one of them being NK cells. The activation and proliferation of antiviral NK cells by locally produced IL-2 might interfere with spread of the virus and hinder the therapeutic efficacy, thus highlighting the importance of effective anti-viral agents that could be used in combination with approaches that indirectly induce high levels of NK cells. Ruxolitinib represents an appealing approach in this respect as it is already FDA and

EMA approved for the treatment of intermediate or high-risk myelofibrosis (Mascarenhas and Hoffman 2012). According to a recently published report, transient blocking of anti-viral NK cells with small molecule inhibitors, including ruxolitinib, can enhance helper-dependent adenovirus (HD-Ad) mediated gene delivery in an *in vitro* co-culture system with human cells (Ankathatti Munegowda and Hu 2015). In Study IV, we found that ruxolitinib potentiates oncolytic adenovirus *in vivo*, despite the observation that adenovirus was completely resistant to IFN-mediated inhibitory effect *in vitro*. This led us to speculate that ruxolitinib affected immune cells such as NK cells which contribute maintenance of anti-viral resistance. Moreover, the discontinuation of ruxolitinib resulted in slow reconstitution of anti-viral state, suggesting that recovery of cellular component had occurred.

As the results depicted in Study IV represent the only *in vivo* studies of ruxolitinib in context of oncolytic viruses (at least to our knowledge), further studies are warranted. Clinical studies with ruxolitinib have suggested that impaired NK cell function may be linked to increased virus infection rate in myelofibrosis patients (Schonberg et al. 2015a), raising concerns about opportunistic virus infections. Nevertheless, ruxolitinib-related effect on NK cells have been reported reversible and patients who have discontinued the therapy have experienced recovery of NK cell levels to normal values (Schonberg et al. 2015b). Finally, a phase II trial found that ruxolitinib can extend the survival of pancreatic cancer patients who have high levels of the inflammation marker C-reactive protein in their blood (Hurwitz et al. 2015), highlighting the possible utility of ruxolitinib and other similar small molecule inhibitors as immunomodulators in cancer therapy.

As described in Study II, combination treatment with virus and T-cells had appealing benefits such as the capacity to induce systemic immunity and to increase repertoire expansion of anti-tumor T-cells. On the other hand, the combinatorial approach was unable to cure mice, probably because mice – like most rodents – are non-permissive for human adenovirus replication (Jogler et al. 2006). As discussed, we hypothesize that

improved efficacy in solid tumors could be achieved if adenoviral replication would be coupled with expression of virus-produced cytokines to enhance T-cell function even further. Furthermore, the importance of tumor cell oncolysis cannot be underestimated as this may lead to continuous viral spread, constant danger signal in the tumor and debulking of high tumor mass, which has been previously linked to impairment of tumor-specific T-cells (Prato et al. 2013). Interestingly, a recently developed model based on semi-permissive Syrian Golden hamsters allowed assessment of these aspects of oncolytic adenovirus and, similarly to mouse data, showed enhanced anti-tumor efficacy when combined to adoptive T-cell therapy (Siurala et al. 2016). Further studies will determine whether this anti-tumor effect can be enhanced even further with cytokine-armed oncolytic adenoviruses and subsequently result in equal or even better efficacy than regimens containing pre- and post-conditioning therapies.

In conclusion, successful implementation of oncolytic immunotherapy necessitates overcoming critical barriers such as premature viral clearance by anti-viral mechanisms and impairment of TIL function by tumor-level immunotolerance. The results presented in this thesis provide further clues on how to hit the immunotherapeutic ‘sweet spot’ in terms of immunomodulation and complement the ongoing efforts to translate the success of preclinical studies into patient benefit.

6 FUTURE PERSPECTIVES

Cancers related to chronic mutagenic exposure (such as tobacco smoking in lung adenocarcinoma or UV light in malignant melanoma) carry frequent mutations (Alexandrov et al. 2013b). Interestingly, these cancer types seem most amenable to immunotherapies such as checkpoint inhibitors (Snyder et al. 2014, Rizvi et al. 2015). However, the prevalence of somatic mutations differs greatly between and within different cancer types (Alexandrov et al. 2013b), indicating that simplistic categorization into "less mutated" or "less immunogenic" tumors based on a specific cancer type does not exclude the possibility of benefiting from cancer immunotherapy.

A typical solid tumor contains 30-70 mutations that possess the capability to function as *de facto* tumor-specific neo-antigens (Vogelstein et al. 2013b). As most of these mutations affect intracellular proteins, mutant residues must be presented in the context of HLA in order to elicit anti-tumor responses. Based on theoretical predictions, typical breast or colorectal cancer has been estimated to contain 7-10 mutant proteins that can bind to a HLA type of a given individual (Segal et al. 2008). Recently, significant effort has been put into directing T-cell activity towards these neo-antigens that represent the "Holy Grail" of cancer immunotherapy, as their expression is limited to the tumor cells eliminating the risk of off-target toxicities. Encouragingly, preliminary mouse and human data has shown that mutant peptides can be successfully used in cancer vaccine platforms (Sampson et al. 2010, Castle et al. 2012). Thus, the discovery and utilization of neo-antigens represent the next level of cancer immunotherapy and approaches capable of inducing neo-antigen targeted immune responses, such as oncolytic immunotherapies, may be in the forefront of this progress.

Despite decades of continuous research and development, curable immunotherapies are still scarce. However, new technologies and improved understanding of tumor microenvironment are paving the way for next generation of cancer treatments. The new

era of oncology, consisting of personalized medicine, cancer immunotherapy and combinatorial approaches, has already provided proof-of-concept results. The fast-growing field of cancer immunotherapy continues to show promise and may eventually enable effective treatment and even cures of cancers resistant to conventional therapies. At the very least, novel combinations of passive and active immunotherapies represent significant improvements in the treatment of a variety of solid cancers provided that critical barriers such as intrinsic and adaptive resistance mechanisms are overcome.

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